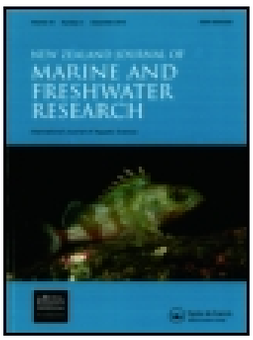

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Faecal streptococci as faecal pollution indicators: a review. Part I: Taxonomy and enumeration

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Abstract The faecal streptococci are the most commonly used alternative or adjunct to coliform bacteria as faecal pollution indicators. They are a group of coccoid bacteria, naturally inhabiting the gut of warm-blooded animals and humans. Their potential as faecal pollution indicators was recognised in the 1890s, but it was 50 years before the accumulated knowledge of their taxonomy and sanitary significance, together with the development of isolation and enumeration methods, allowed their practical application in water pollution research and management. There is still much disagreement over the classification of the group. Many workers now place some species in a separate genus, *Enterococcus*, and it is likely that this revised nomenclature will be incorporated into the next edition of *Bergey's Manual*. Of the enumeration methods available (plate count, MPN, and membrane filtration), the mE-EIA membrane filtration method, which recovers enterococci (a subset of faecal streptococci) is probably the most important, because it is the basis of current United States Environmental Protection Agency recommendations on bathing water standards. However, there appears to be no universally accepted "best method" for the isolation of faecal streptococci from natural waters. Biochemical and serological identification methods are commercially available, and research has begun into the use of DNA-based techniques for identifying faecal streptococcal strains and faecal sources.

Keywords faecal streptococci; enterococci; taxonomy; enumeration; review

INTRODUCTION

Indicating faecal pollution of water

It has long been recognised that water contaminated with faecal material constitutes a disease risk if used for drinking (e.g., the study of typhoid fever by William Budd in 1856: Scarpino 1971), gathering of edible shellfish (e.g., Banks 1917) and recreation (e.g., Simons et al. 1922). However, because a wide variety of micro-organisms are water-transmissible pathogens and tend to be present in polluted water intermittently and/or in low concentrations, methods for their detection and enumeration are often complex and expensive. Alternative organisms that are consistently present in faecal material, survive reasonably well in water compared to pathogens, and are easier to detect, have therefore become widely used as faecal pollution "indicators".

The most commonly used indicator organisms are the coliform bacteria, including their subset, the

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faecal coliforms. They have been extensively investigated, and the literature on their sanitary significance and enumeration has been reviewed by many authors, including Geldreich (1966), Bonde (1977), and Pyle (1981). However, it is now widely acknowledged (e.g., IAWPRC 1991) that no single indicator group or species can be expected to provide a satisfactory indication of disease risk in all situations. Accordingly, there is an increasing amount of investigation into alternative indicators. These include *Clostridium perfringens* (reviewed by Bonde 1977; Cabelli 1977), *Pseudomonas aeruginosa* (reviewed by Bonde 1977; Hoadley 1977), bifidobacteria (e.g., Evison & James 1975; Resnick & Levin 1981) and bacteriophages (reviewed by IAWPRC 1991). However, the most commonly considered alternative or adjunct to the coliforms is the group of bacteria known as the faecal streptococci.

Faecal streptococci have been reported as possible pollution source (human versus animal) indicators (Geldreich & Kenner 1969; Feachem 1975; Oragui & Mara 1981; Rutkowski & Sjogren 1987). This potential, together with the recent adoption by the United States Environmental Protection Agency (USEPA) of enterococci (a subset of the faecal streptococci) as indicators of recreational water quality (USEPA 1986), has led to a closer examination of this group by water managers and public health authorities in New Zealand (see Part II of this review). However, because they are not yet widely used in this country, analytical staff in laboratories concerned with water treatment, wastewater treatment, water pollution monitoring, and public health, often lack access to useful background information on their sanitary significance, classification, and the various methods of enumeration. Part I of this paper reviews the literature on these issues; further information on specific topics is available in the reviews by Barnes (1976), Facklam (1976), Clausen et al. (1977), Skinner & Quesnel (1978), Schleifer & Kilpper-Bälz (1987), Murray (1990), and Ruoff (1990).

History

The potential of intestinal streptococcal organisms to act as pollution indicators was first recognised late last century by Lawes & Andrewes (1894) and Houston (1900). Winslow & Hunnewell (1902) also observed that these organisms were consistently present in the faeces of all warm-blooded animals and in water contaminated with faecal material. Around this time, Thiercelin (1899) also described a new Gram-positive diplococcus, which was termed "entérocoque" on account of its intestinal origin. Thiercelin & Jouhaud (1903) proposed the generic name "Enterococcus" to describe this type of organism, the type species being *Enterococcus proteiformis*. However, Andrewes & Horder (1906) subsequently renamed the species *Streptococcus faecalis*, because of its ability to form chains, and this nomenclature has persisted until recently.

There was also early recognition of differences in biochemical characteristics of streptococci from animal and human faeces. Gordon (1904, 1905) noted that streptococci from human faeces fermented mannitol but not raffinose. In contrast, streptococcal isolates from cows and horses fermented raffinose but not mannitol. These differences were confirmed by Winslow & Palmer (1910). Andrewes & Horder (1906) also found that streptococci from human sewage fermented lactose, whereas those from horse manure did not.

In spite of this initial interest, practical application of faecal streptococci as pollution indicators had to await a more comprehensive characterisation of these organisms, which enabled a better differentiation of faecal from non-faecal isolates. Foremost among these characterisation studies was the biochemical work of Orla-Jensen (1919) and Sherman (1937), and the serological classification of Lancefield (1933). These investigations led to a renewal of interest in faecal streptococci, as is apparent from the work of Suckling (1943) who listed four attributes which make them suitable as faecal pollution indicators:

(1) their occurrence in relatively high numbers in the excreta of humans and other warm-blooded animals;

(2) their presence in wastewaters and known polluted waters;

(3) their absence from pure waters, virgin soils, and other environments having no contact with human and animal life; and

(4) their persistence without multiplication outside the animal body.

After 1940, laboratory methodology associated with the recovery and enumeration of faecal streptococci in water and wastewater developed rapidly, starting with the suggestion by Mallman (1940) that lactose broth could be used in a most probable number (MPN) procedure for streptococcal enumeration. According to Deibel (1964), over 30 different isolation media were formulated in the 1950s and early 60s. During this period, there was also further progress on streptococcal taxonomy and the beginning of systematic studies on their distribution, incidence, and significance in water and food.

CLASSIFICATION AND DEFINITIONS

Terminology

Streptococci

The term "streptococci" has been used to refer to all members of the bacterial genus *Streptococcus*. Recent large-scale taxonomic studies have resulted in a revision of the classification of this group that is still on-going. The paragraph below is an amalgam of a number of descriptions, including those given in the 1974 edition of *Bergey's Manual of Determinative Bacteriology* (Buchanan & Gibbons 1974), the 1986 edition of *Bergey's Manual of Systematic Bacteriology* (Sneath et al. 1986), and the review by Murray (1990).

Streptococci are Gram-positive cocci having spherical or ovoid cells with cell division in one plane resulting in pairs or chains of varying length. They are non or rarely motile. They are aerotolerant anaerobes, lacking cytochromes and catalase, but possessing superoxide dismutase. They ferment carbohydrates to produce mainly lactic acid. Their optimum growth temperature is about 37°C.

Although there have been suggestions that the streptococci should be divided into three separate genera—*Streptococcus*, *Enterococcus*, and *Lactococcus* (Kilpper-Bälz et al. 1982; Schleifer & Kilpper-Bälz 1984)—the 1986 edition of *Bergey's Manual* has named a single genus, *Streptococcus*. On the basis of convenience, rather than strict taxonomy, *Bergey's Manual* also has the streptococci organised into six groups. Streptococci that are regularly recovered from faeces occur in two of these groups: the "enterococcus group" and a miscellaneous group called "other streptococci". This classification is based on that of Jones (1978) and differs from that of Bridge & Sneath (1983), which recognised 28 different groups.

Bergey's enterococcus group includes four species that are found in the faecal microflora of warm-blooded animals and two subspecies that are only associated with plants. However, subsequent authors have extended the group, giving separate species status to *Bergey's* subspecies and recognising a further six species which have been isolated from a variety of sources (Ruoff 1990). The species described in *Bergey' Manual* (Sneath et al. 1986), are: *S. faecalis* and *S. faecium*, which are both found in humans and most other animals; *S. avium*, which is characteristically found in birds, but also occurs in other animals, including humans (Sneath et al. 1986); *S. gallinarum*, which occurs principally in birds, but has also been isolated from a human clinical specimen

Fig. 1 Definition of the terms "faecal streptococci", "Group D streptococci" and "enterococci", based on the streptococcal species belonging to each group (after Clausen et al. 1977).

(Ruoff 1990). *Bergey's* subspecies *S. faecium* subsp. *casseliflavus* has been isolated from plants, soil, and silage (Collins et al. 1984), and *S. faecium* subsp. *mobilis* from grass silage (Sneath et al. 1986).

Two streptococci found in large numbers in faeces are included in *Bergey's* miscellaneous group. *S. bovis* is found in the faeces of ruminants, often as the predominant faecal streptococcus (Oragui & Mara 1981), but can also occur in the faeces of other animals and humans, in clinical specimens, and in dairy products (Sneath et al. 1986). *S. equinus* is the predominant streptococcus in the faeces of horses (Sneath et al. 1986).

Previous systems of classification, and species not recognised in the 1986 *Bergey's Manual* (such as *S. durans*) are still commonly used in the literature and will be briefly described here.

Faecal streptococci

This group consists of species that are recovered from faeces in significant numbers. These species are usually considered to be *S. faecalis*, *S. faecium*, *S. avium*, *S. gallinarum*, *S. bovis*, and *S. equinus*. Some authors include *S. mitis*, and *S. salivarius*—inhabitants of the nasopharyngeal tract and usually grouped with the "oral streptococci". Although normally considered to be rare in faeces, Moore & Holdeman (1974) found these two oral species in high concentrations in human faeces.

Fig. 1 displays a convenient grouping of faecal streptococci species.

Group D streptococci

Lancefield (1933) divided the streptococci into serological groups based on the antigenicity of certain carbohydrates. Lancefield's Group D possesses the antigen glycerol teichoic acid. Streptococci that posses the Group D antigen are those generally considered to be faecal streptococci—*S. faecalis*, *S. faecium*, *S. avium*, *S. gallinarum*, *S. bovis*, and *S. equinus*. *Streptococcus avium* also contains the Group Q antigen and therefore belongs to both Group D and Group Q.

Enterococci

The enterococci include *S. faecalis*, *S. faecium*, *S. durans* (not recognised by some authors), and related biotypes. According to Clausen et al. (1977), the enterococci are those species of faecal streptococci that can:

- grow at both 10°C and 45°C;
- survive at 60°C for at least 30 min;
- grow at pH 9.6;
- grow in the presence of 6.5% sodium chloride;
- reduce 0.1% methylene blue in milk.

A slightly different set of tests is recommended by Murray (1990) for the identification of enterococci in a clinical laboratory, namely, those species of faecal streptococci that:

- exhibit resistance to the antibiotic vancomycin;
- do not produce gas from glucose;
- react with streptococcal Group D antiserum;
- are bile-esculin positive;
- hydrolyse PYR (pyrrolidonyl-ß-naphthylamide);
- grow in 6.5% NaCl broth;
- grow at both 10°C and 45°C.

Viridans streptococci

The viridans group contains the non-enterococcal faecal streptococci; *S. bovis* and *S. equinus* were placed in this group by Sherman (1937), and *S. mitis* and *S. salivarius* are normally also considered to be viridans streptococci (Deibel 1964).

Group Q streptococci

Streptococci containing Lancefield's Group Q antigen are found in the faeces of humans and other warm-blooded animals, especially chickens. *Streptococcus avium* is characteristically found in the faeces of chickens and occasionally in the faeces of humans, dogs, and pigs. The Group D antigen is also found in *S. avium*, indicating that the species properly belongs in the faecal streptococci and that there is a relationship between Group D and Group Q organisms.

Classification into species

The biochemical characteristics of a group of mannitol fermenting, heat-resistant streptococci from faecal sources were examined in detail by Orla-Jensen (1919), and divided into three species: *S. faecium*, *S. glycerinaceus*, and *S. liquefaciens*. In the first major review of the physiological characteristics of the faecal streptococci, Sherman (1937) recognised that *S. glycerinaceus* was identical to the *S. faecalis* described by Andrewes & Horder (1906). Sherman proposed four divisions of streptococci: the enterococcus, pyogenic, lactic, and viridans divisions. The enterococcus division, which closely matched the serological Group D proposed earlier by Lancefield (1933), contained four species: *S. faecalis*, *S. liquefaciens*, *S. zymogenes* , and *S. durans*.

In the Sherman scheme, *S. faecium* (Orla-Jensen 1919) was not designated a separate species because it was considered to be identical with *S. faecalis*. However, *S. faecium* was established as a species distinct from *S. faecalis* by Skadhauge (1950), Shattock (1955), and Barnes (1956).

The status of *S. durans* is still unresolved. Some authors have considered *S. durans* to be a variety of the species *S. faecium* (Jones 1978; Bridge & Sneath 1983). However, Farrow et al. (1983) concluded, from biochemical, menaquinone, fatty acid, and DNA analyses on Group D streptococci, that *S. faecalis*, *S. faecium*, *S. durans*, and *S. avium* were all distinct species.

Sherman (1937) placed *S. bovis* and *S. equinus* in the viridans group of the faecal streptococci. Recent DNA investigations (e.g., Kilpper-Bälz et al. 1982; Farrow et al. 1983) and biochemical studies (Bridge & Sneath 1983) appear to confirm a distant relationship between *S. bovis* and *S. equinus* and members of the enterococcus division (Fig. 1). However, the presence of the Group D antigen in *S. bovis* and *S. equinus* has led to disagreement on their relationship to the enterococci (Jones 1978). *Bergey's Manual* (Sneath et al. 1986) considers that serology should only be considered as one of many properties used to classify streptococci, with no particular importance compared to other criteria.

Classification into sub-species

Some authors have recognised several faecal streptococci subspecies (or varieties). The possibility of *S. faecalis*, *S. liquefaciens*, and *S. zymogenes* being varieties of the same species was raised by Sherman (1937). Three subspecies of *S. faecalis* were also recognised by Deibel et al. (1963), Hartman et al.

(1966), and Facklam (1972): *S. faecalis* var. *faecalis* — distinguished by its inability to both liquefy gelatin and produce beta haemolysis; *S. faecalis* var. *liquefaciens*—also non-haemolytic but capable of gelatin liquefication; *S. faecalis* var. *zymogenes*—recognised by its beta-haemolytic reaction. However, Jones et al. (1972) considered that the haemolysis and proteolysis reactions were insufficient justification for separating these three strains, and considered them all to be *S. faecalis*. This view was given some support by the work of Jacob et al. (1975), who found that the haemolytic ability of *S. faecalis* var. *zymogenes* was plasmid encoded, suggesting that this characteristic may be unstable.

Some authors (e.g., Deibel 1964; Hartman et al. 1966; Kalina 1970; Jones et al. 1972) have considered *S. durans* to be a sub-species of *S. faecium*. Facklam (1972) also considered that the production of a yellow pigment on 5% sucrose agar by a plant-associated *S. faecium* isolate warranted the creation of another *S. faecium* subspecies: *S. faecium* var. (now subsp.) *casseliflavus*, a distinction accepted in *Bergey's Manual* (Sneath et al. 1986). A further plant-associated isolate, *S. faecium* subsp. *mobilis*, has also been recognised in the *Bergey's Manual*.

With the advent of DNA-based classification systems, subspecies have tended to be accorded species status (e.g., *S. durans* and *S. casseliflavus*) as part of an overall revision of streptococcal nomenclature. However, subspecies are still commonly referred to in biochemical classification systems, such as the USEPA procedure (Fig. 2). The USEPA scheme includes *S. salivarius* but not *S. mitis* and accords *S. durans* separate species status. It also recognises *S. faecalis* subspecies *zymogenes* and *liquefaciens* but not *S. faecalis* var. *faecalis* (this is considered to be equivalent to *S. faecalis*) or *S. faecium* var. *casseliflavus*.

Revised nomenclature

From the results of DNA hybridisation studies, Schleifer & Kilpper-Bälz (1984) proposed that *Streptococcus faecium* and *Streptococcus faecalis* be transferred to the genus *Enterococcus*, becoming *Enterococcus faecium* and *Enterococcus faecalis*, respectively. Using biochemical and genetic data, Collins et al. (1984) also proposed that *Streptococcus durans* and *Streptococcus avium* be placed in the genus *Enterococcus* to become *Enterococcus durans* and *Enterococcus avium*, respectively. Recently, it has been suggested that twelve species be included in the genus *Enterococcus* (Ruoff 1990).

Schleifer & Kilpper-Bälz (1984) maintained that their DNA hybridisation studies showed that *Streptococcus faecium* and *Streptococcus faecalis* are only distantly related to *Streptococcus bovis* and *Streptococcus equinus*. It may be inferred from these findings that these authors agreed with the current placement of the latter two species in the genus *Streptococcus*.

Standard Methods (APHA 1989) uses the name *Streptococcus* and recognises six species: *S. faecalis*, *S faecium*, *S. avium*, *S. gallinarum*, *S. bovis*, and *S. equinus*. *S. durans* is not recognised.

New species have been recognised in the DNA-based process of streptococcal nomenclature revision (Farrow & Collins 1985; Collins et al. 1986). Some of these, such as *E. mundtii and E. hirae*, account for atypical or unclassifiable strains isolated from natural and polluted environments by workers in the 1950s and 1960s. The new nomenclature is likely to be recognised in the next edition of *Bergey's Manual* but, for the purposes of this review, the classification in the 1986 edition will be adhered to, with all the faecal streptococci being placed in the genus *Streptococcus*.

ISOLATION AND ENUMERATION

History

The acceptance of faecal streptococci as valid and useful faecal indicators was closely linked to the development of isolation and enumeration methods. In spite of the early observations of Houston (1900), Winslow & Hunnewell (1902), and Winslow & Palmer (1910), a lack of selective media for streptococci prevented practical application of their observations. A renewed interest in the group as pollution indicators followed the development of a most probable number (MPN) method (Mallman & Seligmann, 1950). The development of membrane filtration and plate count methods followed and, more recently, there have been developments in fluorescent antibody techniques, biochemical species identification systems, and DNA-based identification systems. Methods for the isolation and enumeration of faecal streptococci have been reviewed by Hartman et al. (1966), Levin et al. (1975), Barnes (1976), and Clausen et al. (1977).

Most Probable Number

The MPN or multiple tube dilution (MTD) method can be used to enumerate both faecal streptococci and enterococci. Authorised procedures have been

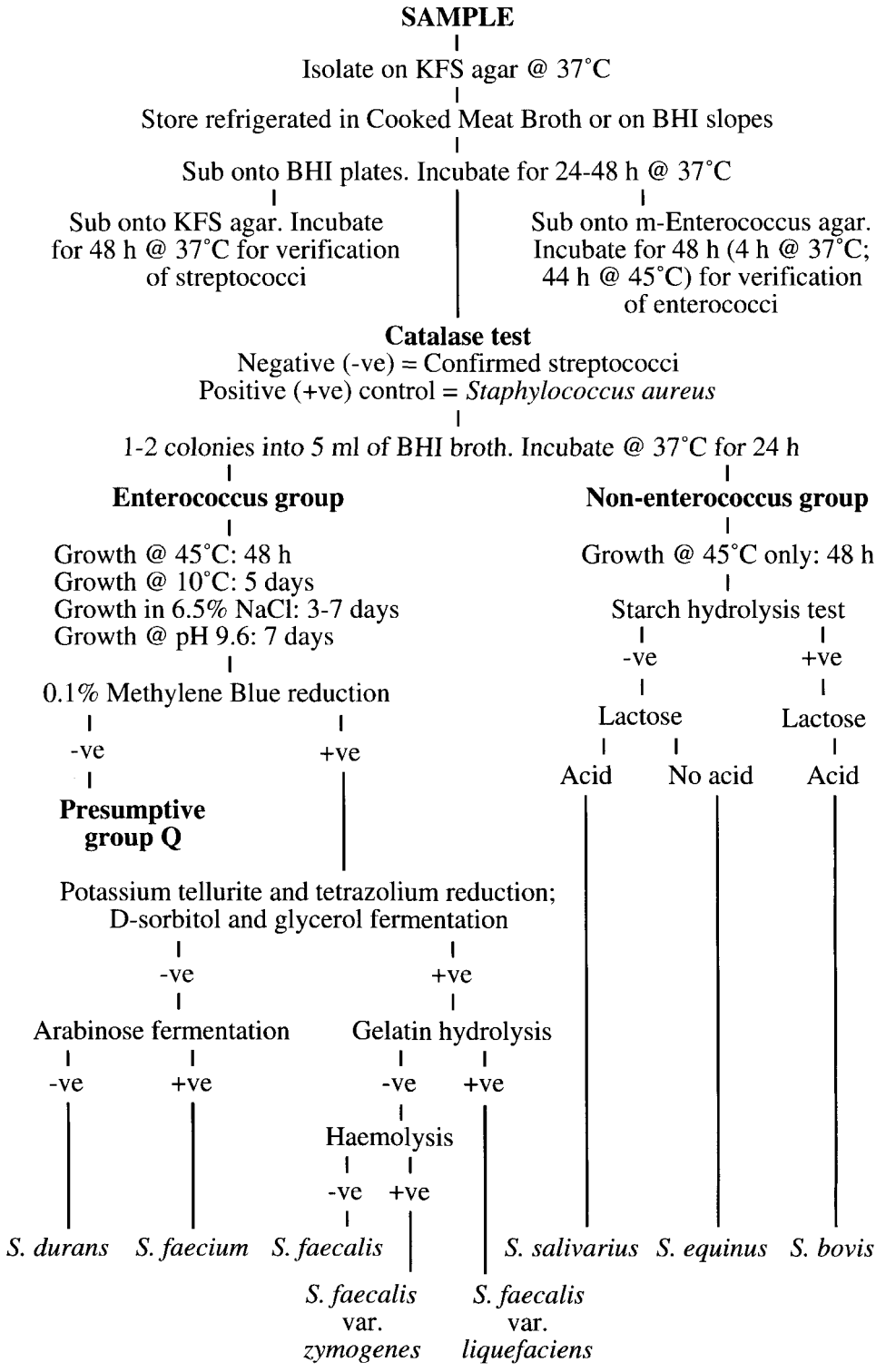

Fig. 2 A faecal streptococci species identification procedure (Bordner et al. 1978).

published in *Standard Methods* (APHA 1989) for faecal streptococci and enterococci and by the World Health Organization (WHO 1982) for faecal streptococci. The APHA methods are recommended for use with raw and chlorinated effluents, sediments, and both fresh and marine waters. The WHO method makes no mention of suitability for sea water. Methods given by both authorities have presumptive isolation in azide dextrose broth (AD) as the first step but differ in their methods of confirmation. *Standard Methods* (APHA 1989) also gives a procedure to obtain an enterococcus count following enumeration of faecal streptococci.

The use of azide lactose broth for MPN was first suggested by Mallman (1940). Hanja & Perry (1943) developed the SF medium and Winter & Sandholzer (1946) described presumptive and confirmatory media which use sodium azide and/or high concentrations of NaCl as inhibitors. The procedure of Mallman & Seligmann (1950) that employed azide dextrose medium was later modified by Litsky et al. (1953, 1955), who reduced the false positive problem by confirming the positive AD tubes in a second medium, ethyl violet azide (EVA) broth.

The AD-EVA procedure involves inoculation of dilutions of sample into replicate tubes of AD broth. Positive tubes exhibit turbidity following 24–48 h incubation at 35–37°C. These tubes are confirmed by transfer to EVA broth where a positive reaction is indicated by the formation of a purple "button", or dense turbidity, after 48 h incubation at 35–37°C. The faecal streptococcus count is estimated from the positive EVA tubes using an MPN table. Various other broths have been recommended for the recovery of faecal streptococci, including Kenner faecal streptococcus (KF) broth (Kenner et al. 1960) and Pfizer selective enterococcus (PSE) broth (Buck 1972). However, the AD-EVA method is probably the most commonly used liquid medium system, and is recommended by WHO (1982). According to Clausen et al. (1977), the method is specific for the enumeration of enterococci, but Volterra et al. (1986) and WHO (1982) consider it to recover faecal streptococci.

Standard Methods (APHA 1989) recommends presumptive isolation in AD broth for 24–48 h followed by confirmation by streaking a portion of growth to plates of PSE agar that are then incubated for 24 h at 35°C. To obtain an enterococcus count, positive colonies can be subcultured to tubes of brain heart infusion (BHI) broth and 6.5% NaCl broth. Growth at 45°C (in BHI broth) and in 6.5% NaCl confirms that the colony is from the enterococcus group.

A method to enumerate enterococci directly from AD broth has been presented by Donnison (1992). A loopful of growth is streaked onto segmented membranes, which are laid on mE agar and subsequently transferred to esculin iron (EIA) agar (Levin et al. 1975). This method has been found to give an equivalent count to the membrane filter mE-EIA procedure and can be used for turbid fresh and marine waters.

Membrane filtration

Standard Methods (APHA 1989) recommends the membrane filtration procedure for the enumeration of faecal streptococci, including enterococci, in fresh and marine waters. The procedure involves drawing a known volume of liquid sample through a membrane filter with a 0.45 μm pore size. Bacteria present in the liquid are retained on the filter surface and the filter is then transferred to a selective medium for colony development. The number of colonies counted on the membrane is taken to be equivalent to the number of streptococcal cells present per volume of sample.

Compared to the most probable number method, membrane filtration produces results more quickly, allows a larger volume of sample to be tested, allows colonies to be counted directly, and allows the immediate biochemical testing of isolated colonies. However, filtration may not be used for highly turbid samples and is not recommended for chlorinated effluent.

Slanetz & Bartley (1957) first reported a method for the enumeration of faecal streptococci (termed enterococci) by a membrane filtration procedure. Kenner et al. (1961) described a membrane filter (KF) medium which increased the isolation frequency of enterococci as well as permitting the growth of non-enterococci. Isenberg et al. (1970) reported a plating procedure for the quantification of Group D streptococci which was based on the esculin reaction. The recoveries of these and other procedures have been compared by Sabbaj et al. (1971) and Switzer & Evans (1974).

Until recently, KF agar was probably the most commonly used faecal streptococci membrane filtration medium. Faecal streptococci appear on this medium as pink to dark red colonies after incubation at 35°C for 48 h. Pavlova et al. (1972) reported that the medium recovered high proportions of faecal streptococci with low numbers of false positives. False positive recoveries have included *Pediococcus cerevisiae* and *Lactobacillus plantarum* (Kenner et al. 1960), but the most common is probably *Staphylococcus aureus* (Mossel et al. 1957). Some

authors (Slanetz & Bartley 1964; Switzer & Evans 1974) have reported the failure of KF medium to support the growth of *S. bovis* and *S. equinus*. However, others (Kenner et al. 1960; Geldreich & Kenner 1969; Pavlova et al. 1972; Daoust & Litsky 1975; Brodsky & Schiemann 1976) have found that KF agar will support the growth of these species. Hall et al. (1963) suggest that, although recovery rates for enterococci are high on KF medium, they are low for *S. bovis* and *S. equinus*. The recovery of *S. mitis* and *S. salivarius* on KF medium has been demonstrated (Kenner et al. 1960; Daoust & Litsky 1975) and Yoshpe-Purer (1989) reported that the medium supported the growth of marine vibrios, making it unsuitable for use in marine waters. It should be noted that KF medium is no longer recommended in *Standard Methods* (APHA 1989).

Daoust & Litsky (1975) demonstrated that Pfizer selective enterococcus (PSE) agar can be adapted for use with membrane filtration. When the membrane is overlaid with PSE medium, faecal streptococci colonies can be distinguished by the presence of dark brown halos indicative of esculin hydrolysis. Esculin hydrolysis is considered to be a very reliable means of separating the Group D from other streptococci (Facklam & Moody 1970; Bayne et al. 1983). However, Brodsky & Schiemann (1976) reported that 95% of the colonies that developed on membranes incubated on PSE (i.e., without a PSE overlay) were identified as Group D streptococci and, therefore, that esculin hydrolysis was not an essential identification criterion. However, Clausen et al. (1977) questioned the species identification methods used in this work.

Recovery rates of faecal streptococci on PSE appear to be equivalent to those on KF medium (Pavlova et al. 1972; Daoust & Litsky 1975; Brodsky & Schiemann 1976; Levin et al. 1975) and PSE also recovers *S. bovis* and *S. equinus* (Nowlan & Deibel 1967; Pavlova et al. 1972; Daoust & Litsky 1975). However, Pavlova et al. (1972) found that PSE produced a higher percentage of false positives than KF medium.

Several media have also been developed for the isolation and enumeration of enterococci by membrane filtration. The first and, until recently the most widely used, was m-Enterococcus medium (Slanetz & Bartley 1957). However, there is now some doubt as to whether this medium recovers faecal streptococci or enterococci. Clausen et al. (1977) gave the m-Enterococcus incubation temperature as 35°C over 48 h. This method is recommended in the 17th and 18th editions of *Standard Methods* (APHA 1989; 1992) for enumerating faecal streptococci in fresh and marine waters. However, the Ministry of Housing and Local Government (1969) maintains that this procedure produces large false positive counts and recommends incubation at 37°C for 4 h, followed by a further 44 h at 44 – 45°C. It appears that *S. bovis* and *S. equinus* are recovered with low efficiency on m-Enterococcus medium (Slanetz & Bartley 1964; Shuval et al. 1973; Daoust & Litsky 1975). *Standard Methods* (APHA 1989, 1992) recommends the use of mE agar, followed by transfer to EIA for enterococcus enumeration. It should be noted that mE agar is the medium developed by Levin et al. (1975), and should not be confused with m-Enterococcus agar, which has a different formulation. The descriptions in *Standard Methods* (APHA 1989, 1992) of the methods for enterococcus and faecal streptococci enumeration are rather confusing. For example, it is recommended that all light and dark red colonies on m-Enterococcus agar (i.e., for faecal streptococcus enumeration) should be counted as enterococci.

Other media formulations and incubation procedures for faecal streptococci have been proposed for specific situations. These include methods for faecal streptococci recovery from sea water and the selection of non-enterococci. Although membrane filtration is not recommended for chlorinated effluents, Lin (1974) increased membrane counts to equal MPN values by enrichment with bile broth or by extending the membrane incubation period from 48 to 72 h. Rutkowski & Sjogren (1987) developed a medium designated M2 to help distinguish between human and animal pollution sources. M2 supported the growth of a wider selection of streptococci than is normally associated with the faecal streptococci, but the authors reported that over 90% of their isolates on this medium belonged to Lancefield's Group D.

Plate count

A tentative pour plate (plate count) method was recommended in *Standard Methods* (APHA 1985) as an alternative procedure to membrane filtration when dealing with highly turbid samples containing few faecal streptococci (sample aliquots are pipetted into Petri dishes and mixed with either KF or PSE agar tempered at 45°C). However, this method was removed from the subsequent edition of *Standard Methods* (APHA 1989). The World Health Organisation (WHO 1982) recommends the use of m-Enterococcus agar for use with pour plates, spread plates, or membrane filtration techniques for enumerating faecal streptococci. They recommend

incubation at 44°C for 48 h, with resuscitation at 37°C for 2–4 h for samples likely to contain stressed bacteria.

Sea water analysis

Buck (1972) evaluated PSE broth for the detection of faecal streptococci in sea water by an MPN method. Levin et al. (1975) reported a preliminary investigation in which the confirmed faecal streptococci recoveries by a modified PSE procedure exceeded those of the KF method by an order of magnitude. However, background growth on the modified PSE was a problem.

To limit the background growth associated with the modified PSE method, Levin et al. (1975) developed a two-stage membrane filtration procedure suitable for marine waters and selective for enterococci. This procedure involved initial recovery on mE agar with confirmation on separate esculin-iron agar plates. The authors reported that the ten-fold improvement (reported with the modified PSE method) over KF agar was retained, background growth was substantially eliminated, and c. 90% of the colonies recovered were confirmed as enterococci. No *S. bovis*, *S. equinus*, *S. mitis*, or *S. salivarius* were recovered with this procedure.

The mE-EIA medium was used on samples in the Adriatic Sea (Volterra et al. 1986) and found to give counts uncomplicated by background colonies, unlike KF and M-enterococcus agars. However, when the enterococcus colonies were further investigated, it was found that 30% of them were *Aerococcus* spp. These workers expressed a preference for KF (not autoclaved) and M-enterococcus agars, both incubated for 48 h at 37°C. However, Yoshpe-Purer (1989), in a study which did not include mE-EIA agar, found that KF was unsuitable for samples from Israeli beaches (it supported the growth of *Vibrio* spp.), but that M-enterococcus agar was satisfactory. These rather contradictory findings support the view of Volterra et al. (1986), who concluded that there is no highly recommended procedure available for the enumeration of faecal streptococci or enterococci in sea water and that accompanying bacterial flora influence all procedures. In addition, recent research has shown that intestinal bacteria become stressed in aquatic environments and enter a "viable but non-culturable" state, from which they cannot be recovered on standard selective media (Grimes et al. 1986). This has now been shown to occur for *S. faecalis* in drinking water (Byrd et al. 1991).

The mE-EIA method was used in the epidemiological studies conducted by the USEPA (Cabelli 1980; Cabelli et al. 1983) which led to the 1986 USEPA recommendation that enterococci should become the basis of marine recreational water standards (discussed in Part II of this review). The mE-EIA method is described in detail in USEPA (1985) and in *Standard Methods* (APHA 1989) where it is recommended for use in both fresh and marine recreational waters. Pagel & Hardy (1980) found that it was also suitable for effluents.

Selective methods for non-enterococci

While methods for the isolation and enumeration of enterococci are well developed, particularly those based on membrane filtration, fewer methods are available for the selection of non-enterococci. However, media have been developed for the improved recovery of *S. bovis*. Switzer & Evans (1974) reported that enterococcosel broth (the same formulation as PSE, minus the agar) recovered almost 50% more *S. bovis* than previously described media. Absorbent pads soaked in the broth were used for membrane filtration. Oragui & Mara (1981) described a membrane-Bovis agar (m-BA) selective for *S. bovis* enumeration when incubated anaerobically. This medium was later modified by the authors by reducing the amount of sodium azide inhibitor (Oragui & Mara 1984).

Identification of species

Several procedures have been outlined for the identification of faecal streptococci species based on biochemical reactions. The most widely accepted procedures are probably those developed by the USEPA (Bordner et al. 1978), as shown in Fig. 2. However, when current USEPA schemes for speciating faecal streptococci were evaluated by Speidel & Hekmati (1981), using standard streptococcal cultures obtained from the American Type Culture Collection (ATCC), these authors found that the test schemes failed to arrive at the species stated by the ATCC in six of the nine standard organisms. The problem areas included carbohydrate fermentation reactions, haemolytic reactions, tellurite reduction, and excessive time and media requirements. Possibly as a result of this study, an identification procedure with only two carbohydrate tests and without tellurite or haemolytic tests has been recommended by *Standard Methods* (APHA 1989).

Other procedures have been documented, such as a constricted tube system for differentiation of Group D streptococci (Takeguchi & Simpson 1979). A miniaturised biochemical technique for the

identification of animal streptococcal isolates was also described by Molitoris et al. (1985) and reported to give good agreement with conventional methods.

Several streptococcal identification systems are now produced commercially. The API-STREP system has been compared with conventional biochemical methods by Waitkins et al. (1981) who reported that Lancefield's Group D and the viridans streptococci gave 100% correlations. The Rapid Strep system (DMS Laboratories, New Jersey), was evaluated by Facklam et al. (1984) who found that, of the Group D streptococci, the system correctly identified 98% of enterococci and 95% of non-enterococci, but correct identifications for the viridans group ranged from 85% for the common species to 10% for less common species.

Fluorescent antibody techniques

The first useful specific Group D antiserum was produced by Pavlova et al. (1972) who pooled sera produced by a variety of strains. Cross reactions with staphylococci and non-Group D streptococci were eliminated by treating smears with trypsin before staining with fluorescent antibody. Pugsley & Evison (1975), using commercial Group D antisera, combined this method with a modified membrane filtration technique, to enable enumeration of faecal streptococci in water. Abshire & Guthrie (1971) also reported a slide technique specific for the detection of *S. faecalis* in water (the antiserum used did not react with *S. faecalis* var. *liquefaciens*).

DNA-based techniques for identification of faecal streptococci strains

Restriction endonuclease analysis

The aim of restriction endonuclease analysis (REA) is to enable the comparison of the "genetic fingerprints" of different strains of bacteria. When the DNA of a bacterium is extracted and treated with a restriction endonuclease enzyme, the DNA is cleaved at specific sites. The resulting fragments can be separated according to size by agarose gel electrophoresis, so that a series of bands representing DNA fragments of different molecular weights is produced. This band series constitutes a unique and reproducible fingerprint for that organism. The method has found application in epidemiological and taxonomic studies in several genera (Robinson et al. 1982; Razin et al. 1983; Collins & DeLisle 1985).

The use of the REA method to separate animal from human-derived faecal streptococci has been investigated by Hastie (1990). REA fingerprints of faecal streptococci isolated from four human volunteers were examined, and identical banding patterns were frequently observed for isolates from the same human individual. However, no fingerprint(s) characteristic of human faecal streptococci (i.e., common to two humans) could be identified. Similarly, a small number of bovine streptococcal isolates generated a diverse range of banding patterns. Even closely related strains of the same species (from the NZCDC Type Culture Collection) exhibited different fingerprints. It was concluded that the REA method was too sensitive to be useful in distinguishing human from animal faecal streptococcal isolates. However, the technique may have application in environmental studies as an accurate method of confirming the identity of specific streptococcal tracers.

Colony hybridisation

DNA-DNA hybridisation aims to determine the degree of homology between two separate strands of DNA. The technique has been well utilised in taxonomic investigations of the faecal streptococci (e.g., Farrow et al. 1983; Knight et al. 1984; Schleifer & Kilpper-Bälz 1984), and has also found application in situations where a genetic probe can detect the presence of DNA from a micro-organism of interest, such as in epidemiological surveys (Grimont et al. 1985) and in investigations of soil bacteria (Holben et al. 1988).

In the microbiological analysis of water, colony hybridisation involves the capture of bacterial cells from a water sample on a nitrocellulose or nylon membrane filter followed by incubation on a selective medium (Amy & Hiatt 1989). The filter is removed and the adherent cells are lysed. The bacterial DNA is denatured with a sodium hydroxide solution to form single strands which are baked onto the filter. Labelled, denatured probe DNA is then incubated with the DNA immobilised on the filter, so that the hybridisation reaction between complementary strands may occur. When a radio-labelled probe is used, hybridised molecules are detected by autoradiography. Probes may also be labelled with non-radioactive compounds, such as biotin, which is usually linked to a colorimetric enzyme assay detection system, streptavidin-alkaline phosphatase being the most commonly used.

The colony hybridisation method has several potential advantages over standard biochemical identification procedures: (1) it is the most direct and complete method of examining homology between

two organisms, since it tests the relatedness of two nucleotide sequences; conventional biochemical tests measure only 10% of the genetic capability of a bacterium (Harvey & Pickett 1980); and (2) it is quick to perform and a large number of colonies can be screened at one time. The method is also compatible with existing membrane filter techniques. However, to date, it appears that the colony hybridisation technique has not been applied to the identification of faecal streptococci in water samples.

SUMMARY AND CONCLUSIONS

1. The faecal streptococci are a group of coccoid bacteria naturally inhabiting the gut of warm-blooded animals and humans. Their potential as faecal pollution indicators was recognised over 100 years ago, but it was 50 years before the accumulated knowledge of their taxonomy and sanitary significance, together with the development of isolation and enumeration methods, allowed their practical application in water pollution research and management.
2. There is much disagreement over the classification of the group. As noted in the principal bacterial classification text—*Bergey's Manual* (Sneath et al. 1986)—this will continue until new data from taxonomic studies become available. Although the nomenclature based on the genus *Streptococcus* as recognised by *Bergey's Manual* (Sneath et al. 1986) is used in this review, many workers now place some species in a separate genus, *Enterococcus* (e.g., Collins et al. 1984; Schleifer & Kilpper-Bältz 1984) and it is likely that this revised nomenclature will be incorporated into the next edition of *Bergey's Manual.*
3. Many methods are available for the enumeration of faecal streptococci, including enterococci, by MPN and membrane filtration, and for the enumeration of faecal streptococci by pour and spread plates. Of these, the mE-EIA method is probably the most important, because it is the basis of current USEPA recommendations on bathing water standards. However, there appears to be no universally accepted "best method" for the isolation of faecal streptococci from natural waters. Recovery rates may be affected by the "non-culturability" of parts of the population and by interferences from accompanying background flora.
4. Biochemical and serological identification methods for faecal streptococci are commercially available, and research has begun into the use of DNA-based techniques for identifying faecal strepto-cocci strains and faecal sources.

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