Characterization of haemolytic enterococci isolated from oral infections

CARL-ERIK NORD & TORKEL WADSTRÖM

Department of Oral Microbiology, Karolinska Institute and Department of Bacteriology, National Bacteriological Laboratory, Stockholm, Sweden

Nord, C.-E. & Wadström, T. Characterization of haemolytic enterococci isolated from oral infections. *Acta Odont. Scand.* 31, 387—393, 1973.

Ninetytwo strains of haemolytic enterococci were isolated from patients with different oral infections — apical periodontitis and pulpitis — and identified by physiological, biochemical and serological tests. All strains produced at least one haemolytic toxin which lysed rabbit erythrocytes. Most strains produced extracellular esterase, protease, and a bacteriolytic enzyme. No lecithinase, lipase, DNAse, amylase or staphylolytic enzyme were observed in the strains investigated.

Keywords: Enterococci; periodontitis; pulpitis; microbiology

C-E. Nord, Department of Bacteriology, National Bacteriological Laboratory, S-105 21 Stockholm, Sweden

The group D streptococci are part of the normal human flora and are usually one of the major bacterial species recovered from the oral cavity, the pharynx and the alimentary canal (*Deibel*, 1964). Enterococci are also involved in different human infections, such as urinary tract infections (*Freedman*, 1963), subacute bacterial endocarditis (*Cherubin & Neu*, 1971), septicemia (*Bengtsson et al.*, 1972) and oral infections (*Möller*, 1966). However, at present very little is known about the mechanism by which this species exerts its tissue damage on the host. Enterococci produce extracellular proteins, such as proteases and haemolysins, which may be toxic to human and animal tissues (*Keys et al.*, 1972). Enterococci have been found in many samples from endodontic infections and members of this genus are considered to be pathogens in these infections (*Engström & Frostell*, 1964). No systemic study has been carried out to characterize haemolytic enterococci isolated from infections in the oral cavity. The purpose of the present investigation was to characterize oral haemolytic enterococci by biochemical, physiological and serological tests and to investigate the ability to produce different extracellular toxins and enzymes.

MATERIAL AND METHODS

Bacteriological specimens from patients with oral infections (periodontitis apicalis and pulpitis; International Statistical Classification of Diseases, Injuries and Causes of Death, WHO 1966) were taken with sterile charcoaled paper-points from the

Received for publication, June 15, 1973.

apical region through the root canal of the teeth. The operation field was always shielded by rubberdam and sterilized by washing with 35 per cent hydrogen peroxide for 5 minutes and 10 per cent iodine tincture for 1 minute. The samples were transported to the laboratory in a liquid storage medium VMG III (*Möller*, 1966) and cultured for 18 hours at 37°C in two liquid and on two solid media: 1) dextrose broth (nutrient broth supplemented with 10 per cent serum and 1 per cent glucose), 2) thioglycollate broth (Difco), and 3) two rabbit blood agar plates (Difco) with 5 per cent defibrinated rabbit red blood cells washed once with saline. One plate was incubated aerobically and the other anaerobically in H_2 with 5 per cent CO_2 in anaerobic jars (*Frostell*, 1957). Samples giving pure culture of haemolytic enterococci were selected for this study.

Microbiological tests. The strains were tested for the following reactions: Catalase production (*Thomas*, 1963); oxidase production (*Kovacs*, 1956); motility (*Tittsler & Sandholzer*, 1936); oxidation/fermentation of glucose (*Hugh & Leifsson*, 1953); growth at 10°C and 45°C (*Cowan & Steel*, 1970); growth at pH 9.6 (*Clarke*, 1953); growth at 6.5 per cent NaCl (*Cowan & Steel*, 1970); growth on potassium-tellurite agar (*Cowan & Steel*, 1970); growth on 10 per cent and 40 per cent bile agar (*Cowan & Steel*, 1970); hydrolysis of aesculin (*Cowan & Steel*, 1970); hydrolysis of arginine (*Niven, Smiley & Sherman*, 1942); hydrolysis of hippurate (*Hare & Colnebrook*, 1934); liquefaction of gelatin (*Cowan & Steel*, 1970); production of acid from arabinose, maltose, trehalose, raffinose, salicin, glycerol, mannitol, sorbitol, sucrose and glucose (*Cowan & Steel*, 1970).

Serology. Grouping sera for groups A, B, C, D, and G were purchased from Difco. Extraction of the antigen was carried out with hot formamide according to *Fuller* (1938). The suspension was used as the undiluted antigen for immunoelectroosmophoresis (IEOP). IEOP was carried out in 1.5 per cent (w/v) nobel agar on glass slides 20 × 10 cm (in a 1 mm thick layer). The buffer in the gels and the electrode vessels was 0.05 M sodium phosphate, pH 7.0. Wells, 2 mm in diameter, were cut in parallel rows 1 cm apart. Twentyfour pairs of wells were cut on each plate. The antigen (100 μl) was placed in the well close to the anode and the grouping sera (100 μl) in the other. Electrophoresis was carried out in an apparatus constructed for quantitative immunoelectrophoresis according to *Laurell* (1965). After electrophoresis at 15 V for 60 minutes, the plate was examined for precipitin lines and reexamined after staining with coomassie brilliant blue R 250. The results were confirmed by the microplate technique for immunodiffusion by *Wadsworth* (1962).

Enzyme and toxin analysis. Production of haemolysin was detected on plates containing blood agar base (Difco) and erythrocytes from rabbit, sheep, human and horse (5 per cent final conc. v/v) washed once with saline. Tests for the production of various proteins were performed on nutrient agar plates (Difco) to which the following substrates were added: The production on protease was studied on agar containing 15 per cent (v/v) of sterile skimmed milk (*Brown & Scott*, 1970). Elastase was assayed on elastin agar plates (*Sbarra, Gilfillan & Bardawil*, 1960). Tributyrin agar (lipase activity) was prepared after sonication of the substrate according to *Hugo & Beveridge* (1962). Lecithin agar (lecithinase activity) contained 5 g of egg lecithin per litre agar

medium (*Wretlind et al.*, 1973). Esterase activity was detected on agar containing indoxylbutyrate (*Holt*, 1971). Amylase was assayed on agar containing blue starch (Phadebase, Pharmacia, Uppsala, Sweden). Bacteriolytic activity was determined on agar plates containing heat killed *Micrococcus lysodeikticus* strain NCTC 2665 (*Hawiger*, 1968) or *Staphylococcus aureus*, strain Copenhagen (*Wadström & Hisatsune*, 1970). Positive reactions were read as zones more than 2 mm around the streaks after growth at 37°C for 48 hours.

DNAse activity was determined on DNAse test agar (BBL) according to *DiSalvo* (1958). After 48 hours at 37°C the plates were flooded with normal hydrochloric acid and the positive culture showed a clear zone around the streak.

Chemicals

The carbohydrates and salts used were all of analytical grade. Casein hydrolysate, yeast extract and the agar bases were purchased from Difco, elastin powder was obtained from Sigma Chemical Company, St. Louis, Mo., USA, and indoxyl butyrate was purchased from Koch-Light, Colnbrook, Bucks, England.

RESULTS

In 3141 bacteriological specimens from oral infections, 945 strains of enterococci were found in pure culture. Ninetytwo strains of these enterococci were haemolytic and were further characterized.

Microbiological tests. The isolated strains consisted of ovoid cells elongated in direction of the chain, 0.5—1.2 μm in diameter, occurring mostly in pairs or short chains. The gram reaction was positive. Three strains were motile. The colonies were white to grey, 2—4 mm in diameter, on blood agar plates. All strains grew at 10°C and at 45°C. They all showed visible growth in broth containing 6.5 per cent NaCl, pH 9.6, on 10 per cent and 40 per cent bile agar and on potassium-tellurite agar. All strains fermented glucose and maltose. Ninetyone strains produced acid from salicin, 87 strains from trehalose and 71 strains from sucrose. Arabinose and raffinose were not fermented. Aesculin was split by all the strains tested. Eightynine strains hydrolyzed arginine and hippurate hydrolysis was detected in 44 strains. Gelatin was liquefied by 20 strains. Catalase was present in two cultures but no oxidase activity was observed. The microbiological tests are summarized in Table I.

Serology. The polysaccharide preparations from all the enterococci investigated reacted with the group D antisera. The precipitates were obtained after 30 minutes of electrophoresis. The reaction between the antigen extracts of 23 strains with the antisera is shown in Fig. 1. The precipitates were stained with coomassie brilliant blue R 250 which increased the sensitivity about fivefold and simplified the reading. No cross reactions were obtained with groups A, B, C, and G antisera. The reactions were also confirmed by use of the microplate diffusion agar technique.

Production of enzymes and toxins. Table II shows analysis of the strains for production of haemolysis and enzymes. All strains showed haemolytic activity on erythrocytes from rabbit, 73 strains had activity

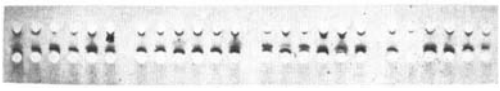

Fig. 1. Immunoelectroosmophoresis of group D antiserum and extracted antigen from 23 oral haemolytic enterococci.

Table I *Microbiological reactions of the oral enterococci tested*

Test	Number of cultures Positive/number of cultures tested
Catalase	2/92
Oxidase	0/92
Motility	3/92
Fermentation	92/92
Antigenic group D	92/92
Growth at 45°C	92/92
Growth at 10°C	92/92
Growth at pH 9.6	92/92
Growth at 6.5 % NaCl	92/92
Resistance to tellurite	92/92
Growth on 10 % bile	92/92
Growth on 40 % bile	92/92
Aesculin hydrolysis	92/92
Arginine hydrolysis	89/92
Hippurate hydrolysis	44/92
Gelatin liquefaction	20/92
Acid from	
Arabinose	0/92
Maltose	92/92
Trehalose	87/92
Raffinose	0/92
Salicin	91/92
Glycerol	90/92
Mannitol	90/92
Sorbitol	90/92
Sucrose	71/92
Glucose	92/92

Table II. *Production of various haemolysins and enzymes by 92 strains of oral haemolytic enterococci*

Haemolysin activity on erythrocytes from	rabbit	92
	human	73
	horse	67
	sheep	14
Esterase		90
Protease		35
Elastase		22
Bacteriolytic enzyme		70

No production of lecithinase, lipase, DNAse, amylase and staphylolytic enzyme was observed.

on human erythrocytes, 67 strains on horse erythrocytes and only 14 strains on sheep erythrocytes. Ninety strains were found to produce esterase. Thirtyfive cultures were protease producers on casein agar and 22 produced elastase. Seventy strains showed positive reaction on the plates containing *M. lysodeikticus* used for analysis of bacterioloytic activity. No lecithinase, lipase, DNAse, amylase or staphylolytic enzyme were observed in the strains investigated.

DISCUSSION

Enterococci include *S. faecalis* and its varieties (*zymogenes* and *liquefaciens*), *S. faecium* and *S.durans*. Group D streptococci are all those possessing the group D antigen. This includes all enterococci plus *S. bovis* and *S.equinus* (*Barnes*, 1956; *Raj & Colwell*, 1966). The different physiological and biochemical reactions for the streptococci belonging to group D are shown in Table III. As seen from the table the properties of enterococci are markedly distinguished from other streptococci and therefore *Kalina* (1970) has proposed a new genus *Enterococcus* including two species, *E. faecalis* and *E. faecium*.

All our 92 strains investigated grew at 10° and 45°C, in 6.5 per cent NaCl, at pH 9.6, on media containing 40 per cent bile and were resistant to tellurite. All strains fermented glucose and maltose and hydrolyzed aesculin. Most strains fermented sucrose, trehalose, salicin, glycerol, mannitol and sorbitol. No cultures produced acids from arabinose or raffinose. Therefore, we consider that our strains belong to *S. faecalis*. The strains are most closely resembling *S. faecalis var. zymogenes* because the strains were haemolytic.

Table III. *Physiological and biochemical reactions of group D streptococci*

Test	S.bovis	S.equinus	S.durans	S.faecalis	S.faecium
Growth at					
45°C	+	+	+	+	+
10°C	—	—	+	+	+
pH 9.6	—	—	d	+	+
6.5 % NaCl	—	—	+	+	+
Growth on tellurite	—	—	—	+	—
10 % bile	+	+	+	+	+
40 % bile	+	+	+	+	+
Hydrolysis of aesculin	+	+	+	+	+
Arginine	—	—	+	+	+
Hippurate	—	—	d	d	d
Degradation of gelatin	—	—	—	d	—
Acid from					
Arabinose	d	—	—	—	+
Maltose	+	+	+	+	+
Trehalose	d	—	d	+	+
Raffinose	+	—	—	—	—
Salicin	+	+	d	+	+
Glycerol	—	—	+	+	+
Mannitol	d	—	+	+	d
Sorbitol	—	—	—	+	—
Sucrose	+	+	d	+	+
Glucose	+	+	+	+	+
Haemolysis	α/-	α	β/-	β/-	α/β

+ = 80—100 % strains positive; d = 21—79 % strains positive; — = 0—20 % strains positive.

Streptococcus faecalis var. zymogenes was earlier regarded as a separate species, *Streptococcus zymogenes*, but it is believed that the differences are not sufficient to warrant species distinction. *Deibel* (1964) also described that strains belonging to *S. faecalis* show frequent loss of haemolytic and proteolytic activity. The haemolytic action of *S. faecalis var. zymogenes* depends on the kind of erythrocytes used in the plates. *Updyke* (1967) described that 88 per cent of 90 enterococci showed haemolysis on rabbit, horse and human blood agar plates but not on sheep blood plates. We also found that only a small number of strains produced haemolysis on sheep blood. The proteolytic activity of enterococci on gelatin is also a questionable test, since gelatin is not chemically well defined and there is no correlation between gelatin and casein activities. Thirtyfive of our strains tested had activity on casein, but only 20 strains were active on gelatin. For the differentiation of *S. faecalis*, *S. faecium* and *S.durans* determination of esterase activities may be useful, since *S.faecalis* strains possess very active esterases in contrast to the other enterococci (*Lund*, 1965). This observation was confirmed in our study. The lack of catalase is one of the tests employed to differentiate streptococci from staphylococci. However, some enterococci are known to possess catalase activity (*Langston, Gutierrez & Bouma*, 1960) and this was confirmed in our study. The catalase activity is not only restricted to the enterococci, some pedicocci and

lactobacilli have also been found to have this activity. This is an important thing to keep in mind when typing different gram-positive cocci. Three of our strains tested were found to be motile, and strains of enterococci including both *S. faecalis* and *S. faecium*, have been reported to be motile in contrast to other species of streptococci (*Hugh*, 1959). Recently *Amstein & Hartman* (1973) examined the fatty acid composition of different enterococci by gas chromatography. They reported similar fatty acid patterns of *S. faecalis*, *S. faecium* and *S. durans*. However, they also found that a motile streptococcus differed from the other strains by the lack of some fatty acids. We are now going to test our three isolates of motile enterococci for the composition of fatty acids. This may be a useful taxonomic tool for differentiation of some species belonging to enterococci.

Thus no single test is available for rapid diagnosis of different streptococci. The haemolytic activity of streptococci is still the one mostly used. However, several biochemical tests are valuable in confirming the classification of streptococci. The most important test for the recognition of streptococci is the immunologic identification of the group-specific antigen. All enterococci possess the group D antigen. The presence of this antigen is also demonstrated in *S. bovis* and *S. equinus*. The antigen is a tecihoic acid — a polymer of glycerol phosphate containing a high proportion of glucose and alanine ester residues on the sugar. The location of the antigen is associated with the cytoplasm or the cytoplasmic membrane in contrast to the cell wall location of the group antigen in groups A, B, C, and G streptococci (*Krause*, 1972). It is also well-known that the antigen is not always extractable by the Lancefield HCl technique (*Shattock*, 1949) and therefore we used the formamide method (*Fuller*, 1938) for the extraction of the antigen.

The enterococci prodominate in the human flora, while the *S. bovis — S.equinus* species are found in animals. Enterococci occur in rather small numbers in the oral cavity and most of the strains are isolated from the gingival crevice region (*Socransky*, 1970). In a survey looking for group D organisms in oral specimens, we found that nearly all the group D streptococci in oral infections are *S. faecalis* (*Nord & Wadström*, 1973). *Facklam* (1972) recently described subgrouping of group D streptococci isolated from different human infections and found also that most strains were *S. faecalis* with its varieties *zymogenes* and *liquefaciens*. *Engström & Frostell* (1964) pointed out that when root canals are infected with *S. faecalis* the prognosis for successful treatment is impaired. However, little is known about the way in which *S. faecalis* exerts its damaging effects on oral tissues. Therefore in a subsequent paper experimental oral infections with *S. faecalis* in dogs will be reported.

REFERENCES

Amstein, C. F. & Hartman, P. A. 1973. Differentiation of some enterococci by gas chromatography. J. Bact. 113, 38—41

Barnes, E. M. 1956. Tetrazolium reduction as a means of differentiating *Streptococcus faecalis* from *Streptococcus faecium*. J. Gen. Microbiol. 14, 57—68

Bengtsson, E., Strandell, T., Svanbom, M. & Tunevall, G. 1972. Azidocillin treatment of enterococcal septicaemia. Scand. J. Infect. Dis. 4, 143—148

Brown, M. R. W. & Scott, J. H. 1970. A simple diagnostic milk medium for *Pseudomonas aeruginosa*. J. Clin. Path. 23, 172—177

Cherubin, C. E. & Neu, H. C. 1971. Infective endocarditis at the Presbyterian Hospital in New York City from 1938—1967. Am. J. Med. 51, 83—96

Clarke, P. H. 1953. Growth of streptococci in a glucose phenophtalein broth. J. Gen. Microbiol. 9, 350—352

Cowan, S. T. & Steel, K. J. 1970. Manual for the identification of medical bacteria. Cambridge University Press. London

Deibel, R. H. 1964. The group D streptococci. Bact. Rev. 28, 330—366

DiSalvo, I. V. 1958. Deoxyribonuclease and coagulase activity of Micrococcia. Med. Tech. Bull. 9, 191—196

Engström, B. & Frostell, G. 1964. Experiences of bacteriological root canal control. Acta Odont. Scand. 22, 43—70

Facklam, R. R. 1972. Recognition of group D streptococcal species of human origin by biochemical and physiological tests. Appl. Microbiol. 23, 1131—1139

Freedman, L. R. 1963. Pyelonephritis and urinary tract infection, in *Diseases of the Kidney*. (Edited by M. B. Strauss and L. G. Welt) p. 469. Little Brown & Company, Boston USA

Frostell, G. 1957. Bakteriologisk diagnostik vid tandläkarhögskolan i Stockholm. Sv. Tandläk. Förb. Tidn. 49, 229—236

Fuller, A. T. 1938. The formamide method for the extraction of polysacharides from hemolytic streptococci. Brit. J. Exp. Path. 19, 130—139

Hare, R. & Colebrook, L. 1934. The biochemical reactions of hemolytic streptococci from the vagina of febrile and afebrile parturient women. J. Path. Bact. 39, 429—442

Hawiger, J. 1968. Frequency of staphylococcal lysozyme production tested by plate method. J. Clin. Pathol. 21, 390—393

Holt, R. J. 1971. The detection of bacterial esterases and lipases. Med. Lab. Technol. 28, 208—209

Hugh, R. & Leifson, E. 1953. The taxonomic significance of fermentative versus oxidative metabolism of carbohydrates by various gram negative bacteria. J. Bact. 66, 24—26

Hugh, R. 1959. Motile streptococci isolated from the oropharyngeal region. Can. J. Microbiol. 5, 351—354

Hugo, W. B. & Beveridge, E. G. 1962. Quantitative and qualitative study of the lipolytic activity of single strains of seven bacterial species. J. Appl. Bact. 25, 72—82

Kalina, A. P. 1970. The taxonomy and nomenclature of enterococci. Int. J. Syst. Bact. 20, 185—189

Keys, T. F., Sapico, F. L., Tonchon, R., Barenfus, M. & Hewitt, W. L. 1972. Experimental enterococcal endocarditis. Am. J. Med. Sci. 263, 103—109

Kovacs, N. 1956. Identification of *Pseudomonas pyocyanea* by the oxidase reaction. Nature 178, 703

Krause, R. M. 1972. The antigens of group D streptococci, in *Streptococci and streptococcal diseases*. (Edited by L. W. Wannamaker and J. M. Matsen). p. 67—74. Academic Press, New York and London

Langston, C. W., Gutierrez, J. & Bouma, C. 1960. Motile enterococci (*Streptococcus faecium var. motilis var. N*) isolated from grass silage. J. Bact. 80, 714—718

Laurell, C. B. 1965. Antigen — antibody crossed electrophoresis. Anal. Biochem. 10, 358—361

Lund, B. M. 1965. A comparison by the use of gel electrophoresis of soluble components and esterase enzymes of some group D streptococci. J. Gen. Microbiol. 40, 413—419

Möller, Å. J. R. 1966. Microbiological examination of root canals and periapical tissues of human teeth. Akademiförlaget, Göteborg

Niven, C. F., Smiley, K. L. & Sherman, J. M. 1942. The hydrolysis of arginine by streptococci. J. Bact. 43, 651—660

Nord, C.-E. & Wadström, T. 1973. Susceptibility of haemolytic oral enterococci to eight antibiotics *in vitro*. Acta Odont. Scand. 31, 395—399

Raj, H. & Cowell, R. R. 1966. Taxonomy of enterococci by computer analysis. Can. J. Microbiol. 12, 353—362

Sbarra, A. J., Gilfillan, R. E. & Bardawil, W. A. 1960. A plate assay for elastase activity. Nature 188, 322—323

Shattock, F. 1949. The streptococci of group D; the serological grouping of *Streptococcus bovis* and observations on serologically refractory group D strains. J. Gen. Microbiol. 3, 80—92

Socransky, S. S. 1970. Relationship of bacteria to the etiology of periodontal disease. J. Dent. Res. 49, 203—222

Thomas, M. 1963. A blue peroxide slide catalase test. Mon. Bull. Minist. Hlth. Lab. Serv. 22, 124—125

Tittsler, R. P. & Sandholzer, L. A. 1936. The use of semisolid agar for the detection of bacterial motility. J. Bact. 21, 575—580

Updyke, E. L. 1957. Laboratory problems in the diagnosis of streptococcal infections. Public Health Lab. 15, 78—80

Wadström, T. & Hisatsune, K. 1970. Bacteriolytic enzymes from *Staphylococcal aureus*. Purification of an endo-β-N-acetylglucosaminidase. Biochem. J. 120, 725—735

Wadsworth, C. 1962. A microplate technique employing a gel chamber compared with other micro- and macroplate techniques for immune diffusion. Int. Arch. Allergy. 21, 131—137

Wretlind, B., Hedén, L., Sjöberg, L. & Wadström, T. 1973. Production of enzymes and toxins by hospital strains of *Pseudomonas aeruginosa* in relation to serotype and phagetyping pattern. J. Med. Microbiol. 6, 91—100