THE CLASSIFICATION OF ANAEROBIC COCCI AND THEIR ISOLATION IN NORMAL HUMAN BEINGS AND PATHOLOGICAL PROCESSES

BY

C. G. A. THOMAS AND R. HARE

From the Department of Bacteriology, St. Thomas's Hospital Medical School, London

(RECEIVED FOR PUBLICATION MAY 21, 1954)

In a previous paper (Hare, Wildy, Billett, and Twort, 1952) a scheme employing fermentation of carbohydrates and organic acids was put forward for the classification into six groups of the anaerobic cocci derived from human beings.

There is at present extremely little information as to the occurrence of anaerobic cocci on the mucous and skin surfaces of normal human beings. This, together with a study of strains from a number of infective processes, forms the subject of this paper.

Material and Methods

Source of Strains.—The following were chosen.

Nose.—Woollen swabs on straight applicators were employed to obtain samples from the anterior nares of 54 normal persons, medical students and technicians, during the first three months of 1953.

Throat.—Swabs from the tonsillar region of 15 normals were examined during the first six months of 1953.

Tonsils.—Single tonsils from 80 persons, mostly children, were washed with sterile saline immediately after excision. They were seared on the lateral surface, incised from this aspect with a sterile knife, and samples obtained with a platinum loop.

Faeces.—Stools were obtained from 50 in-patients, mostly young adults, suffering from some condition in which the alimentary tract was not considered to be involved.

Vagina.—High vaginal swabs were taken during the first three months of pregnancy from two series of patients, the first of 48 persons during the summer of 1948, and the second of 45 persons during the summer of 1953.

Skin.—Swabs moistened with sterile broth were used to take samples from the dorsum of the right wrist of nine persons.

Gums.—A platinum loop was used to take samples from the gum margin in the region of a

lower canine tooth from nine persons with apparently healthy gums.

External Auditory Meatus.—Dry swabs on straight applicators were used to investigate nine persons.

Strains from Infections.—Three groups of infections were chosen.

Puerperal Pyrexia.—Eight strains from cases in this hospital, 48 isolated by Dr. R. M. Calman, two by Dr. L. R. Janes, one by Dr. A. Beck, and one by Dr. A. Cunliffe were examined. No patient was severely infected.

Infections of the Respiratory Tract.—Strains from 16 cases of chronic bronchitis, bronchiectasis, lobar pneumonia, asthma, pulmonary collapse and empyema, from four abscesses of the lung removed by lobectomy, and from 14 specimens of fluid obtained during bronchoscopy in suspected cases of carcinoma of the lung were sent us by Dr. Weatherley Mien and Mr. N. R. Barrett.

Other Infections.—Strains from cases with an infected frontal sinus, gangrenous appendix, appendix abscess, sinus following appendicitis, brain abscess, operation wound, infection of the foot, and two from the blood in bacteraemia were sent us by Dr. G. Martyn, Dr. Anne Gibson, Dr. L. R. Janes, Dr. J. Bamforth and Dr. R. M. Fry, Dr. J. Pinniger and Dr. H. Loewenthal.

Media for Isolation of Strains from Normal Persons.—Previous experience had shown that a suitable solid medium for the cultivation of anaerobic cocci contained Evans peptone 2.0%, Difco yeast extract 0.2%, agar 2.0%, and 5% horse blood. Since sodium oleate, 0.01%, has no inhibitory effect on these organisms but may promote the growth of strains of Groups III and IV, it was added to the medium (Wildy and Hare, 1953).

Although many specimens were plated in parallel on the same medium containing 40 units per ml. Polymyxin E to inhibit Gram-negative bacilli

(previous work having shown that at this concentration the antibiotic has no effect on the anaerobic cocci (Hare *et al.*, 1952)), there was no evidence that anything was gained by this procedure.

Isolation of Strains.—Whenever possible the specimens from normal persons were plated within a few minutes of being taken. The plates were incubated anaerobically for 48 hours in a McIntosh and Fildes jar. Subcultures of each variety of colony found on such plates were made in parallel on two blood agar plates, one of which was incubated anaerobically and the other aerobically. After 48 hours Gram-stained films were made of those strains growing on the anaerobic plate, but which failed to grow on the aerobic plate. Cocci isolated in this way, irrespective of their Gram reaction, were subcultured twice more from single colonies on to plates incubated anaerobically and aerobically. Provided there was no growth on the plates incubated aerobically on the three occasions, the strain was accepted as an anaerobic coccus and preserved by transferring a fairly heavy inoculum to Robertson's meat medium, incubating for 24 hours, and then storing in the refrigerator.

Many of the strains from patients with infections were isolated by other workers on their routine media. Those isolated in this hospital were obtained from blood agar plates made with Lemco broth. Care was taken on receipt of such strains to determine their purity and that they fulfilled our criteria in regard to anaerobiosis.

Classification of the Organisms Isolated

In the original scheme put forward by Hare *et al.* (1952) fermentation of five carbohydrates (glucose, fructose, maltose, sucrose, and galactose) and five organic acids (pyruvic, lactic, citric, malic, and tartaric) was employed, but in a subsequent paper Wildy and Hare (1953) showed that, although 0.01% sodium oleate had no detectable effects on Groups I, II, V, and VI, strains of Groups III and IV could ferment one or other substance much more actively in its presence. Hare *et al.* (1952) also showed that the presence of a sulphur compound was essential for the production of gas by Group I strains.

For these reasons the basal medium employed for classification contained 0.01% sodium oleate and 0.1% sodium thioglycollate in 2% Evans peptone, with 0.2% Difco yeast extract.

Each batch was made in the same way, the peptone, yeast extract, and sodium thioglycollate being dissolved in distilled water, brought to the boil, and filtered through hard filter paper at once. Sodium oleate, 0.01%, was then added. To this medium 1%

of one or other of the five carbohydrates or the sodium salts of lactic, tartaric, and citric acids were added. Because the sodium salts of pyruvic and sodium malic acids were not available, the acids themselves in concentrations of 1% were added. The pH of the medium was adjusted to 8.0 and 2.5 ml. placed in 5 in. $\times$ $\frac{1}{2}$ in. tubes. Melted vaseline was run on the surface and the tubes sterilized by steaming on three successive days. The same medium containing 1% pyruvic acid but no added oleate was employed for growth of the strains before inoculation into the test media.

Of the 233 strains referred to in this paper, 63 had been classified by Hare *et al.* (1952) by the same methods but in media without added oleate. These strains were not retested in the media described above, but there is no reason to suppose that different results would have been obtained, since the very great majority were members of groups unaffected by fatty acid. The remaining 170 strains were all tested in oleate-containing media. For this purpose about 0.1 ml. of a 48-hour culture in the inoculating medium described above was inoculated into each of the test media. The cultures were incubated for seven days. The length of gas column produced by displacement of the vaseline plugs upwards was measured, the opacity of the culture on the Brown and Kirwan (1915) scale ascertained, and the pH determined with indicators on porcelain tiles. Representative strains from each of the groups were included in any series of tests with new batches of media. These tests showed that, provided the media be made in the same way, different batches behave in an almost identical manner.

If fermentation of the carbohydrates occurred the pH was depressed from the initial 7.6–7.8 to 5.6–6.4, and if gas was formed the vaseline plug was displaced upwards, usually by 10 mm. or more. When the organic acids fermented the pH was seldom depressed very much, but large quantities of gas were produced, and it was on this feature that ability to ferment organic acids was assessed.

Deep cultures of all the strains were made in 1.5% plain agar in broth containing 2.0% peptone, 0.2% yeast extract, 1.0% pyruvic acid, and 0.01% sodium oleate, and the distance between the surface and the upper edge of growth measured, in order to provide some data as to the degree of anaerobiosis required by each strain.

Films were also made from the inoculating culture, stained by Gram's method, and the size and arrangement of the cocci of each strain ascertained.

Results

Table I summarizes the behaviour of members of each of the groups in the oleate-sulphur-containing media described above. It will, however, be observed that in addition to Groups I–VI, already described by Hare *et al.* (1952), three more groups have been added. This was necessitated by the fact that a sufficiently large number of

TABLE I
SCHEME OF CLASSIFICATION EMPLOYED

Group	Substrate Employed											Habit of Growth
	Control	Glucose	Sucrose	Maltose	Fructose	Galactose	Pyruvate	Maltate	Citrate	Tartrate	Lactate	
I (<i>Strep. putridus</i>)	—	AG	—	AG	AG	—	G	—	—	—	—	Chains
II	—	—	—	—	—	—	G	—	G	—	—	Masses
III	—	AG	AG	AG	AG	AG±	G	—	—	—	—	"
IV	—	—	—	—	A	—	G±	—	—	—	—	"
V	—	—	—	—	—	—	G	G	—	—	—	"
VIa	—	A	A	A	A	A	—	—	—	—	—	Chains
VIb	—	A	A	A	A	A±	—	—	—	—	—	Masses
VII	—	A	—	—	A	—	—	—	—	—	—	Masses and chains
VIII	—	AG	—	—	AG	—	G	—	—	—	—	Masses
IX	—	—	—	—	—	—	—	—	—	—	—	"

A = acid, G = gas, in media containing 0.01% sodium oleate, 0.1% sodium thioglycollate, and 1% of the substance.

TABLE II
THE PRESENCE AND CLASSIFICATION OF THE ANAEROBIC COCCI IN NORMAL HUMAN BEINGS

Site	No. of Persons Examined	No. of Persons Carrying Anaerobic Cocci	Percentage of Individuals Sampled who were Carriers	No. of Representatives of Each Group	Group										Not Classified
					I	II	III	IV	V	VIa	VIb	VII	VIII	IX	
Nose ..	54	23	42.6	34	0	1	1	8	0	0	3	1	15	0	5
Throat ..	15	10	66.7	12	0	0	0	0	3	1	7	0	0	1	0
Gums ..	9	7	77.8	8	0	0	0	0	6	0	2	0	0	0	0
Tonsils ..	80	38	47.5	43	0	0	0	0	1	5	8	0	0	22	7
Faeces ..	50	5	10.0	6	0	0	0	0	1	0	0	2	0	1	2
Vagina ..	93	22	23.7	29	4	2	7	6	0	0	0	0	1	3	9
Skin ..	9	4	44.4	8	0	0	1	2	0	0	0	1	3	0	0
Ear ..	9	1	11.1	1	0	0	0	0	0	0	1	0	0	0	0
Total ..				141	4	3	9	16	11	6	21	4	19	27	21

strains having similar characteristics was isolated in the course of the work. Thus, of the 233 strains studied in this way, all but 27 could be placed in one or other of the nine groups.

From Table II it is evident that anaerobic cocci are comparatively common on the mucous and skin surfaces of human beings. They were present in the anterior nares of 42.6% of the population sampled, with Groups IV and VIII predominating. In another series of investigations on 95 nurses, our colleague Dr. N. P. L. Wildy isolated Group IV strains from 31. The throat (surface of the tonsils and posterior wall of the pharynx) was studied in only 15 persons, but 66.4% were carriers, the majority of the strains being Group VI. No less than 38 out of the 80 tonsils examined contained anaerobic cocci, with Groups VI and IX predominating. Only about one in four of these tonsils showed pathological changes to the naked eye. The highest carrier rate of all was found in the examination of the gums, seven out of the nine persons yielding these organisms.

In contrast to the nasopharynx, anaerobic cocci would appear to be unusual in the faeces, for only

six strains were isolated from the 50 specimens examined. Only one of these strains was a member of groups previously described (Group V). Although the very low yield may have been due in part to the fact that the medium employed for isolation was only partially inhibitory for the coliforms present and the colonies of anaerobic cocci are not distinctive, very careful search was always made.

The specimens from the vagina were not "normal" in that they were all taken after pregnancy had started, but anaerobic cocci were undoubtedly present. The carrier rate, 23.7%, was somewhat lower than that reported by White (1933) in a previous investigation (30% in the last month of pregnancy), but it is significant that four of the strains were members of Group I (*Strep. putridus*) which is almost certainly the principal cause of puerperal infection by anaerobic cocci.

The surface of the skin of the wrist was studied in only nine persons, but four carried anaerobic cocci. These belonged to groups common in the nasopharynx, suggesting that they come from this area. The external auditory meatus, on the other hand, yielded only one strain.

In specimens from all the areas sampled except the ear, a number of individuals were found to be carriers of cocci of more than one group. An extreme example of this was a sample taken from the skin which gave 12 colonies warranting further study. Two were Group III, three Group IV, two Group VIII, and five could not be allocated to a group but behaved in the same way.

Strains Isolated from Pathological Processes

In addition to the investigation of anaerobic cocci in normal persons, an attempt has been made to ascertain the possible pathological significance of the different groups. The results obtained are given in Table III.

It is evident that, when anaerobic cocci are present *post partum* in the vagina of patients with pyrexia, Group I is by far the commonest variety. Probably because these organisms are sensitive to penicillin, which is used nowadays as soon as pyrexia develops, none of the patients from whom these organisms were isolated were infected with any severity. Nevertheless, very similar organisms have been isolated from severely infected (several fatal) cases by previous workers (Schottmüller, 1910; Harris and Brown, 1929; Colebrook and Hare, 1933; Stone, 1940). But this organism seems to be responsible for other forms of infection besides puerperal fever, since it was also found in an infected wound of the foot, an abscess of the brain, a septic operation wound, and a gangrenous appendix.

Specimens from the respiratory tract, such as material removed at bronchoscopy, lung abscesses

removed by operation, and sputa from chronic lung infections, yielded many strains of anaerobic cocci. It is quite impossible to make any definite statement in regard to the role of these organisms in the pathological conditions from which they were derived. There was certainly evidence of bacterial infection, but other species of organisms besides anaerobic cocci were always present. The anaerobic cocci isolated were members of Groups IV, V, and VI, which appear to be common inhabitants of the normal respiratory tract.

Of the remaining strains four, belonging to Group VIa, came from an infected frontal sinus, an appendicular abscess, a sinus following appendicitis, and the blood of a case with ill-defined pyrexia. Another strain (also from ill-defined bacteraemia) could not be allocated to a group.

Morphology of the Anaerobic Cocci

The majority of the cocci isolated were Gram-positive spheres, 0.6 to 0.8 μ in diameter, showing no particular arrangement under the microscope. But strains of Groups I and VIa grow in the form of chains, as do two of the four strains of Group VII. Strains of Group V are also Gram-positive but only in young cultures. After 24 hours they are almost all Gram-negative. The size of the cocci of Groups III and IV depends on the oleate content of the medium, being 0.7 to 0.8 μ when sufficient quantities are present, but in ordinary media without added oleate they tend to be larger, diameters of 1.0 to 1.6 μ being common (Wildy and Hare, 1953).

TABLE III
CLASSIFICATION OF THE ANAEROBIC COCCI FOUND IN DIFFERENT TYPES OF INFECTION

No. of Patients	No. of Patients with Anaerobic Cocci	No. of Representatives of Each Group	Group										Unclassified
			I	II	III	IV	V	VIa	VIb	VII	VIII	IX	
14	9	10*	0	Respiratory Tract Material Aspirated at Bronchoscopy					1	0	0	0	0
				0	0	2	5	2					
4	2	3*	0	Abscess of Lung Removed by Operation					0	0	0	0	0
				0	0	1	1	1					
16	8	10†	0	Sputum from Chronic Lung Infections					1	0	0	0	0
				0	0	3	5	1					
		60	42	Genital Tract Puerperal Pyrexia					2	0	0	0	5
				5	2	3	0	1					
				Other Forms of Infection									
				0	0	0	4						
Total		92	46	5	2	9	11	9	4	0	0	0	6

* One Group V and one Group VIb isolated from the same specimen. † One patient with Group V and Group VIa; one patient with Group IV and Group VIa.

Degree of Anaerobiosis Required for Growth

Employing deep cultures in agar, measurement of the distance between its surface and the upper edge of growth gave indications of the degree of oxygen tolerance of each strain. In general, this distance was 5–15 mm. and with many strains has remained relatively constant for several years. Contrary to what is usually supposed, very few strains have become capable of aerobic growth with continued subcultivation.

Discussion

Although gas formation, fermentation reactions, and morphology have been suggested by previous workers (notably Prévot, 1948) as suitable criteria for the classification of the anaerobic cocci, attempts to use these tests have generally come to nothing, for it is quite frequently impossible to obtain regular results or to detect any metabolic activity whatever. This is undoubtedly due to the rather peculiar requirements of these organisms, sulphur compounds being required for gas formation by some strains and fatty acids for fermentation by other strains, while only organic acids and not carbohydrates can be attacked by still other strains. There seems no doubt that sufficient amounts of some of these substances may be present as impurities in some batches and absent in other batches of media to nullify the value of any tests in which peptone, agar, or digest broth are employed for the growth of these organisms. But, provided such possible sources of error are borne in mind and care be taken to add the accessory substances known to be necessary, these organisms can not only be classified into nine groups, but members of each of the groups behave in a consistent way when tested in different batches of medium.

It is evident that anaerobic cocci of one or other group are comparatively common commensals in human beings, but that each area tends to possess a rather restricted number of groups. Because they are all sensitive to penicillin, their pathological importance at present is very small. But strains of Group I (*Strep. putridus* Schottmüller) were at one time an important cause of puerperal infection, and this is certainly by far the commonest group found in the puerperal pyrexias occurring nowadays. Group I strains may also occur in non-puerperal forms of suppuration, and from the description of the strains isolated from anaerobic streptococcal myositis by MacLennan (1943) it would appear that some at least are due to Group I strains.

Anaerobic cocci are stated to be common in appendicular abscesses (Altemeier, 1938). We have had no opportunity to verify this, but, of the three strains from infections in this area, one was Group I and two Group VIa. No strains from chronic burrowing ulcer (Meleney and Johnson, 1937), human bite infections (Boland, 1941), and post-operative synergistic gangrene (Brewer and Meleney, 1926) were examined by us. Nor is it possible from the published descriptions to fit the strains isolated by the workers concerned into our classification.

The organisms belonging to Groups II to IX would seem to have less pathological importance. It is, however, possible that, because most of these strains will ferment carbohydrates or organic acids such as pyruvate, they may in some situations so lower the local pH that the local defensive mechanisms cannot cope with other organisms which may also be present. In this way they may prepare the ground without necessarily being pathogenic themselves. They may therefore possess more importance than would at first sight appear.

Summary

Anaerobic cocci are frequently present on the mucous and skin surfaces of normal persons.

Employing fermentation reactions in the presence of sodium oleate and sodium thioglycollate, the majority of strains encountered can be assigned to one or other of nine groups.

A comparatively restricted number of groups predominates in the flora at various sites of the human body. There is reason to believe that strains of Group I (*Strep. putridus*) are the commonest potential pathogens.

In addition to the many colleagues mentioned above who have assisted us by sending strains or specimens, our thanks are also due to Dr. van der Merwe, Mr. Garland, and Mr. Shaw of this hospital, to Dr. H. J. Parish of the Wellcome Research Laboratories for the Polymyxin E, and to Mr. E. Winslade for technical assistance.

REFERENCES

- Altemeier, W. A. (1938). *Ann. Surg.*, **107**, 634.
- Boland, F. K. (1941). *J. Amer. med. Ass.*, **116**, 127.
- Brewer, G. E., and Meleney, F. L. (1926). *Ann. Surg.*, **84**, 438.
- Brown, H. C., and Kirwan, E. W. O'G. (1915). *Indian J. med. Res.*, **2**, 763.
- Colebrook, L., and Hare, R. (1933). *J. Obstet. Gynaec., Brit. Emp.*, **40**, 609.
- Hare, R., Wildy, P., Billett, F. S., and Twort, D. N. (1952). *J. Hyg., Camb.*, **50**, 295.
- Harris, J. W., and Brown, J. H. (1929). *Bull. Johns Hopk. Hosp.*, **44**, 1.
- MacLennan, J. D. (1943). *Lancet*, **1**, 582.
- Meleney, F. L., and Johnson, B. A. (1937). *Surgery*, **1**, 169.
- Prévot, A. R. (1948). *Manuel de classification et de détermination des bactéries anaérobies*, 2nd ed. Masson, Paris.
- Schottmüller, H. (1910). *Mitt. Grenzgeb. Med. Chir.*, **21**, 450.
- Stone, M. L. (1940). *J. Bact.*, **30**, 559.
- White, E. (1933). *J. Obstet. Gynaec. Brit. Emp.*, **40**, 630.
- Wildy, P., and Hare, R. (1953). *J. gen. Microbiol.*, **9**, 216.