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The isolation of a Gram-negative diplococcus similar in morphology to *N. meningitidis* and having an antigenic component identical in group-specificity to Group 'D' meningococcal agglutinating sera (Wellcome Brand).

by

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INTRODUCTION

In addition to the two well known human pathogens, the gonococcus and meningococcus, Cowan and Steel (1965) in their *Manual for the Identification of Medical Bacteria* make mention of three Neisseria types which have been thought to be clinically associated with meningitis in the human subject.

These are *N. flavescens*, originally isolated by Branham in 1930, *N. mucosa*, originally isolated by Von Lingelsheim (see Cowan and Steel, 1965) and other cultures isolated as *Diplococcus mucosus* (McFarlan, 1941; Bray and Cruickshank, 1943), which last mentioned have rather different characters and resemble *Bacterium anitratum*.

Cowan and Steel also state that cultures identified as *D. mucosus* have, on repeated subculture, become bacillary in form and were undoubtedly strains of *Bacterium anitratum*. They also report that *N. mucosa* which was reisolated by Sir Philip Panton from the cerebrospinal fluid of two patients operated upon by him were identified and described by Cowan (1938), but the cultures were lost during the 1939-1945 war. These strains identified by Cowan reduced nitrates, were highly virulent for mice, and produced filtrates which were also toxic for mice in quite small doses. In all these characters they differed from *B. anitratum* and Cowan and Steel dissent from the view that *B. anitratum* is a synonym of *D. mucosus*.

From a patient with meningitis, Reimann and Koucky (1939) isolated a similar organism but did not identify it conclusively (see Cowan and Steel, 1965).

LABORATORY EXAMINATION

Cerebro-spinal fluid aspirated from an 18 year-old female patient who is said to have developed monoplegia of the right leg was received at the Medical Research Institute from the Government hospital Batticaloa on 29.5.1968 for cultural examination. No further details of clinical history were available. The cerebro-spinal fluid received was very turbid. The turbidity could have been most aptly described as being of a 'ginger-beer' turbidity. Smears were made from the specimen, and stained by a modification of Gram's method described by Preston and Morrel (1962).

The direct smear stained by this method showed no pus-cells. The smear examined microscopically revealed gram-negative diplococci in pairs and very occasional gram-positive thick bacilli suggestive of spore-bearing bacilli. The coccal forms were flattened on their contiguous sides and resembled pairs of 'coffee-beans'.

The specimen was innoculated into :—

(1) Brain-Heart Infusion Broth.
(2) Fildes' Broth.
(3) Blood Agar (direct plating).

The culture of cerebro-spinal fluid on the blood agar was incubated in an atmosphere of 10% carbon dioxide. The brain-heart infusion broth and Fildes' broth were incubated aerobically.

After overnight (about 18 hours) incubation of the innoculated media, the culture on blood agar in carbon dioxide showed elevated, whitish, smooth, moist colonies of an opaque nature. Smears made from these colonies and stained as afore (modification of Gram's method) showed gram-negative diplococci as in the direct smear, which was picked into brain-heart infusion broth for purification and enrichment.

The brain-heart infusion broth and Fildes' broth also appeared turbid after overnight incubation. The broths were then plated on to a set of blood agar and McConkey's lactose agar plates respectively. The sub-cultures from the broths on blood agar were incubated in carbon dioxide. The sub-cultures from the broths on McConkey's lactose agar were incubated aerobically.

The sub-cultures made from the brain-heart infusion broth on blood agar showed smooth, moist, whitish colonies (as afore described). Smears made from these colonies revealed the presence of gram-negative diplococci and gram-negative cocco-bacilli.

The subcultures made from the Fildes' broth on blood agar showed gram-positive cocci and occasional gram-negative diplococci. Suspicious colonies from subcultures of the Fildes' broth on blood agar were again re-subcultured on to blood agar and brain-heart infusion broth for purification.

The subcultures from the broths on to McConkey's lactose agar proved to be that of *Klebsiella aerogenes*, and had the following biochemical properties :—

Lactose	Sucrose	Dulcite	Maltose	Salicyn	Glucose
+	+	+	+	+	+
Mannite	**Indole**	**Methyl Red**	**Koser**	**Motility**	**Urea**
+	O	O	+	O	O

+ = acid and gas produced ; ± = acid only ;

O = growth ; — = no growth.

"THE ATYPICAL GRAM-NEGATIVE DIPLOCOCCUS"

The purified subcultures of the Gram-negative diplococcus having a morphology typical to that of *N. meningitidis*, and the cerebro-spinal fluid having a ginger-beer turbidity, characteristic of meningoccal infections, I directly proceeded to do the serological typing of the organism with 'Welcome' agglutinating meningoccal sera. The agglutination was done with colonies of the 'suspicious' organism from blood agar. The organism was seen to give positive agglutination with Group 'D' meningoccal sera. It did not agglutinate with Groups 'A', 'B' and 'C' meningococcal sera. Subsequent subcultures of the organism on blood agar were tending to give more 'mucoid' colonies. It was after this sera-type was established that I proceeded to do the biochemical tests for this organism.

BIOCHEMICAL TESTS

The colonies of the organism had no effect on the blood cells in the medium. Since all Neisseria colonies give the characteristic oxidase test upon application of a solution of tetramethyl p-phenylenediamine, the oxidase test was done as described by Cowan and Steel in their Manual, using a sterile filter paper and sterile capillary glass tubes. The control organism used was *Ps. pyocyanea.*

The organism was oxidase negative, catalase positive; to obtain fermentation with *N. meningitidis* rabbit or human serum should be added to the medium. Accordingly, Hiss' serum sugars, namely, glucose, maltose and sucrose were used. The organism fermented only glucose with acid production both when incubated in carbon dioxide and aerobically. Maltose and sucrose were not fermented. The organism was found to attack the glucose in Hugh and Leifson's medium only oxidatively. There was acid production in the open tube only and not in the paraffin-covered tube.

The morphology of the organism still continued to be diplococcal and the agglutination with Group 'D' meningococcal sera was still positive.

The organism was also found to grow on Lemco broth and Lemco agar at 37°C and also grew in the ordinary peptone sugars fermenting glucose only at the surface.

The organism did not reduce nitrate also, where the nitrate strip was planted in blood agar. Nitrate reducing bacteria growing on the blood agar should reduce the haemoglobin to methaemoglobin.

The organism grew on nutrient agar at 22°C The organism did not at any stage produce any pigment. The organism in a 4-hour old culture in nutrient (Lemco) broth showed typical, *non-motile*, 'coffee-bean' shaped diplococci on a 'hanging drop' examination. The organism was also found to be non-motile at 22°C but motile at 37°C using Craigie's technique at 22°C and 37°C.

The organism was capsulated and non-sporogenous.

BIOCHEMICAL TABLE OF SUGAR FERMENTATION

TABLE I
HISS' SERUM SUGARS.

Glucose	Maltose	Sucrose
±	O	O

TABLE II
Peptone-Sugars

Lactose	Sucrose	Dulcite	Maltose	Salicyn
O	O	O	O	O

Glucose	Mannite	Indole	Methyl Red	Koser	Motility	Urea
±	O	O	O	O	O	(+) at surface only.

\+ = acid and gas produced ; ± = acid only;
O = growth ; — = no growth.
(+) = NH_3

PATHOGENICITY

Broth cultures (12 to 24 hours old) of the organism were injected intraperitoneally into 2 white mice in doses of .1 ml to each mouse, and were found to be fatal to one of them in about 24 hours. Gram-negative diplococci were demonstrated in smears made from the peritoneal fluid and liver surface. There were no gram-negative diplococci in smears made from the heart blood.

DISCUSSION

Zinsser (1960) states "Bacteria do not change from cocci to bacilli or mutate from genera to genera, but profound changes in biologic activity, *antigenicity* and virulence occur within the framework of the species themselves....."

...."A mutation is a spontaneous, undirected change in an organism that results in heritable change. Such variations are considered to be the result of changes at the submicroscopic, chemical level."

In view of the above, it is submitted that this organism which has biochemical properties similar to that of *Bacterium anitratum*, still represents and reveals a serological linkage to the *N. meningitidis* in that it has an antigenic component identical in *type* specificity to the Group 'D' meningococcus. It thus becomes a serotype of *N. meningitidis*. Cowan and Steel in their *Manual for the Identification of Medical Bacteria* have preferred to refer to *Bacterium anitratum* as *Acinetobacter anitratus*.

The organism isolated is oxidase-negative and the Oxidation-Fermentation Test (O.F. Test) showed that it attacks glucose only oxidatively. It grew at 22°C; and on nutrient agar it fermented only glucose with acid production and did not reduce nitrate. It did

not produce pigment and there was no haemolysis on blood agar. It was catalase positive. In so far as the above tests referred to in the second-stage Table for Neisseria, Gemella and *Acinetobacter anitratus*, by Cowan and Steel go, this organism is biochemically *Acinetobacter anitratus*. *Gemella haemolysans* ferments maltose and sucrose in addition to glucose and also shows haemoylsis.

The organism isolated cannot be associated therefore with *Gemella haemolysans*.

On the contrary, it does not share any of these biochemical characteristics with *N. meningitidis* except for the fact that it is catalase-positive, and does not reduce nitrate. The fact of its ability to grow on ordinary basal nutrient agar and its ability to grow at 22°C. shows its divergence in cultural characters with the *Neisseria meningitidis*.

Newly described species of Neisseria, *N. caviae* and *N. animalis* are included in the second-stage table of Cowan and Steel (1965), but both of which are oxidase-positive; *N. caviae* further reduces nitrate and *N. animalis* ferments sucrose also with acid.

As opposed to *N. mucosa* this organism does not reduce nitrate.

As opposed to all Neisseria organisms this organism is oxidase negative.

As distinct from *N. flavescens*, originally isolated by Branham, the organism did not produce pigment and agglutinates with Group 'D' antimeningococcal serum. According to Zinsser *N. flavescens* produced a golden yellow pigment in the colonies. The various strains were 'homologous' but *did not agglutinate* in antimeningococcal serum. Further, *N. flavescens* lacked the ability to ferment the usual carbohydrates. But Cowan and Steel report that "at first none of the strains isolated in Chicago attacked any sugars but after many years in artificial culture our strains, and those kept by Dr. Branham acquired the ability to produce acid from glucose, maltose and sucrose."

It is further stated by Zinsser on the Neisseria group of organisms that the morphologic similarity between members of the group and the presence of common proteins and somatic carbohydrates suggest that all gram-negative cocci originated from a common ancestor. The organisms occur characteristically in pairs with long axes of the oval cells parallel to the line of division, and there is a definite flattening of the adjoining sides making up a pair. In pure cultures, however, only a minority of the organisms will have this characteristic morphologic arrangement.

Man is the only natural host for *N. meningitidis*.

The organisms live in the nasopharynges of apparently normal individuals and are passed from man to man as are pneumococci.

Freshly isolated organisms of Group 'A' and Group 'C' are encapsulated, and have the typical colony form, smooth, moist, elevated, greyish blue colonies appear.

The organisms of Group 'B' usually are non-encapsulated, and the colonies are smaller, rougher, and may develop a yellowish tint.

Antigenic Structure :—

The nucleo-proteins of meningococci are antigenic and toxic, but not specific since antisera prepared with the nucleo-protein fraction react with the other members of the Neisseria group and with pneumococci. The meningococci also contain a somatic polysaccharide which is common to the other neisserias, pneumococci and some strains of klebsiellas. Zinsser then refers to Bergey's Manual (seventh edition) in which the groups are designated, A, B, C and D. Group specificity is determined by a specific capsular polsaccharide in Groups 'A' and 'C' and by a polysaccharide-polypeptide complex in Group 'B'. The specific capsular polysaccharides are not toxic but delay recovery by neutralizing specific antibodies produced by the patient.

Specific types within the groups do occur but these tend to cross agglutinate with each other and even the gonococcus is frequently agglutinated by sera from organisms in Group 'B'. The purified capsular polysaccharides are not antigenic for man and the absorption of Group 'A' antiserum with Group 'A' polysaccharides fails to remove all of the protective antibodies.

This suggests that the specific antigenic fraction is a polysaccharide-protein complex.

In Table 33 of Zinsser's Bacteriology giving the relationship among the various classifications of meningococci, it is stated that the relation of Group 'D' to the other Groups is unknown. Zinsser records that over 1500 strains, isolated from clinical cases of meningitis during World War II, were typed in one central laboratory. Of these 91.6% were Group 'A', 1.6% were Group 'B',5.6% Group 'C', and only 0.2 percent could not be classified.

Since World War II the epidemic Group 'A' organisms have been sharply reduced and the endemic groups 'B' and 'C' have become prevalent.

It is submitted that the organism isolated having antigenic components identical in group specificity with Group 'D', suggests an interesting link, more so because Cowan and Steel also report that cultures of *N.mucosa* identified and described by Cowan (1938) were lost during the 1939-1945 war.

Further, from a patient with meningitis, Reimann and Koucky (see Cowan and Steel, 1965) isolated a similar organism but did not identify it conclusively.

Topley, Wilson and Miles (1966) state that similar strains which do not acidify glucose peptone water-described by De Bord (1939) as *Mima polymorpha* - are also occasionally met with in cerebrospinal fluid or blood. They resemble the Anitratus group morphologically and culturally.

Topley, Wilson and Miles, however, add that many Mima strains have no action on glucose in Hugh and Leifson's medium. They appear to form a somewhat more heterogenous group than anitratus, and it would be wise to defer their classification until they have been compared with the saprophytic achromobacteria.

Diplococcus mucosus differs from the Anitratus group, however, in being Nitrate positive and never showing bacillary forms (Cowan, 1938).

The organism isolated was found to be motile at 37°C. using Craigie's technique. But, according to Lantrop (1961) quoted by Topley, Wilson and Miles, most strains of *A. anitratus*, when examined on thin agar plates poor in nutrients, exhibit a slow gliding or creeping motility, and should hence be considered as myxobacteria.

It will be interesting to find out whether further isolations of *A. anitratus* from human sources would show a serological relationship to meningococci, as has occured in this case.

It is also of significance to note that positive agglutinations of gram-negative diplococci (morphologically resembling *N. meningitidis*) with meningococcal sera should not be taken as conclusively establishing the 'diplococcus' as *N. meningitidis*, until the biochemical tests have been investigated.

CONCLUSION

Cowan and Steel (1965), in their *Manual for the identification of Medical Bacteria* state that *Acinetobacter anitratus* (*Bacterium anitratum*) is often found in human clinical material; it is frequently misidentified as *Diplococcus mucosus* discussed with the neisseria in the history. One of the characteristics of *A. anitratus* is its ability to attack monosaccharides but not higher saccharides in peptone water sugars. When the sugar concentration is raised to 5 or to 10% it can attack lactose.

Cowan and Steel record that most cultures sent to them as *D. mucosus* have, on repeated subculture, become bacillary in form and were undoubtedly strains of *A. anitratus*.

The organism isolated still retains its diplococci morphology, in solid agar and liquid broth inspite of several sub-cultures. It is submitted in conclusion that this organism isolated is *A. anitratus* having antigenic polysaccharide-protein components similar in group specificity to *N. meningitidis* Group 'D', and presents an interesting serological link between the Acinetobacter-genus on the one hand and the Neisseria-genus on the other.

N.B. It is worthy of mention here that another isolation of *Acinetobacter anitratus* from a specimen of sputum received from Ward 5, De Soysa Home for Women, Colombo, on 4.6.1968, also showed positive agglutination with Group 'D' meningococcal sera.

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REFERENCES

BRAY, P. T. & CRUICKSHANK, J. C. (1943).—Meningitis due to Diplococcus mucosus. *Br. med. J.*, **1**, 601-602.

COWAN, S. T. (1938).—Unusual infections following cerebral operations, with a description of *Diplococcus mucosus*. (Von Lingelsheim). Lancet, **2**, 1052-1054.

COWAN, S. T. & STEEL, K. J. (1965).—*Manual for the Identification of Medical Bacteria*. pp. 69 & 77. Cambridge: The University Press.

DE BORD, G. G. (1939).—Organisms invalidating the diagnosis of gonorrhoea by the smear method. *J. Bact.*, **38**, 119-120.

MC FARLAN, A. M. (1941).—*Diplococcus mucosus* Von Lingelsheim : A description of a strain with comments on the systematic position of the organism. *J. Path. Bact.*, **53**, 446-449.

PRESTON, N.W. & MORREL, A. (1962).—Reproducible. Results with the Gram-Stain. *J. Path. Bact.*, **84**, 241-243.

TOPLEY, W.W. C., WILSON, G. S. AND MILES, A. A. (1966).—*Principles of Bacteriology and Immunity*, 5th ed., pp. 787-789. London: Edward Arnold Ltd.

ZINSSER, H. (1960).—*Bacteriology* Ed. SMITH, D. T. AND CONANT, N. F. 12th ed. pp. 60-62, 306-312. New York: Appleton-Century-Crofts, Inc.