A SEROLOGICAL STUDY OF CERTAIN MEMBERS OF THE AEROBIC NON-PATHOGENIC *NEISSERIA* GROUP

By

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INTRODUCTION

The American scheme of classification--as indicated in Bergey's Manual of Determinative Bacteriology (Breed, et al, 1948)--recognizes 6 distinct species of aerobic, non-pathogenic Neisseria. The similar morphology and uniform non-pathogenicity prevailing among these organisms, coupled with conflicting data on description of pigment, colonial structure, and biochemical activity, have raised doubts as to the validity for recognition of this number of separate species. In contrast to this scheme of species differentiation, some British workers (Wilson and Miles, 1946) support the establishment of a single species, Neisseria pharyngis, for all of the saprophytic Neisseria with the possible exception of N. catarrhalis. These divergent views on the systematic relationship of the saprophytic Neisseria emphasize the need for their further characterization.

Although numerous serological investigations have been conducted with the 2 pathogenic members of the genus, serological studies of the non-pathogenic species have been limited. Various investigators have reported that some of the saprophytic Neisseria auto-agglutinate in saline and/or normal sera. Difficulties encountered in attempts to prepare homogenous suspensions of these organisms appear to be the chief reason for a lack of serological information. Nevertheless, several investigators have applied successfully the agglutination technic for the study of Neisseria.

The study reported here is a serological investigation of some non-pathogenic Neisseria to establish any correlation between this and other characteristics.

II

HISTORICAL

The genus Neisseria derives its name from Neisser, who in 1879 observed the gonococcus in pus cells from gonorrheeal patients. In 1887 Weichselbaum isolated the other pathogen of this genus, the meningococcus, from the meningeal exudate of 6 cases of cerebrospinal meningitis, and published further reports on this organism in 1902 and 1905 (Wilson and Miles, 1946). The gram-negative coccus, Micrococcus catarrhalis, was described by R. Pfeiffer in 1896 and subsequently was thoroughly studied in 1902 by Ghon and H. Pfeiffer (Wilson and Miles, 1946). von Lingelshein (1906) encountered a number of gram-negative cocci in the nasopharynx of healthy and diseased persons, and gave descriptions of M. pharyngis siccus, M. pharyngis cinereus, and Diplococcus pharyngis flavus I, D. pharyngis flavus II and D. pharyngis flavus III. Another member of this genus, Neisseria flaveseens, was isolated from the spinal fluid of patients with epidemic cerebrospinal meningitis and was characterized by Branham (1930). With the exception of the gonococcus, the gram-negative cocci are found almost exclusively in the nasopharynx and upper respiratory tract.

von Lingelshein (1906) reported the following fermentation reactions of several species of gram-negative cocci: (1) M. pharyngis cinereus and M. catarrhalis produced no acid in 9 carbohydrates, (2) M. pharyngis siccus, D. pharyngis flavus I and D. pharyngis flavus II fermented the same carbohydrates, namely, glucose, maltose, and fructose, and (3) D. pharyngis flavus III fermented only glucose and

maltose. Elser and Huntoon (1909) examined the saprophytic gram-negative cocci of the nasopharynx for the purpose of characterising organisms which might give rise to diagnostic difficulties of the meningococci. These investigators reported the biochemical activity of the saprophytic Neisseria in 11 carbohydrates, and found fermentation reactions to be reliable data for the separation of the species, M. catarrhalis, M. pharyngis siccus, and 3 groups of chromogenic cocci, designated as chromogenic groups I, II, and III. Gordon (1921), who examined gram-negative cocci from the nose and throat of normal persons and of persons with colds or influenza, proposed 6 sub-groups of chromogenic cocci in addition to the species, M. catarrhalis and M. pharyngis siccus. Wilson and Smith (1928) concluded that fermentation tests did not afford a reliable means of distinguishing between members of the saprophytic gram-negative cocci. From the results of Wilson's (1928) thorough study of a few strains over a long period, and from the study by Wilson and Smith (1928) of a relatively large number of strains it was suggested that the species, -- catarrhalis, flavus, cinereus, mucosus, and siccus --- be grouped together and designated pharyngis.

Although the merits of consolidation of the above species must be recognised, it is essential also that one consider the general agreement among several workers (von Lingelshein, 1906; Elser and Huntoon, 1909; Martin, 1911; and Gordon, 1921) who divided the saprophytic gram-negative cocci partly on their fermentative capacity and partly on their pigment formation and colonial appearances. Significant differences in cultural characteristics, pigment production, and biochemical activity were correlated, and upon these

differences the species listed in Bergey's Manual (Breed et al., 1948) were resolved. Six species of aerobic, saprophytic Neisseria were presented. Some of the differential characteristics were as follows:

(1) N. catarrhalis - non-chromogenic, moist colonies on agar, and no action from any of the carbohydrates.

(2) N. sicca - non-chromogenic, dry crumbly colonies, and acid from glucose, fructose, maltose, and sucrose. Precipitate spontaneously when suspended in normal salt solution.

(3) N. perflava - chromogenic, and acid from glucose, fructose, maltose, sucrose, and mannitol.

(4) N. flava - chromogenic, and acid from glucose, fructose, and maltose.

(5) N. subflava - chromogenic, and acid from glucose, and maltose. Agglutinates in normal rabbit serum.

(6) N. flavescens - chromogenic and no acid from any carbohydrates.

The fermentation of mannite described for N. perflava (Breed et al., 1948) was not confirmed by Pelczar and Nemes (1949) for their strains of pigmented Neisseria which fermented glucose, fructose, maltose and sucrose.

There is general agreement concerning the failure of N. catarrhalis to produce acid in any of the carbohydrates usually employed for biochemical differentiation. Two types of catarrhalis colonies were described by Elser and Huntoon (1909) and 4 types by Gordon (1921). Wilson and Miles (1946) considered the colonial structure of N. catarrhalis subject to variation, and suggested the formation of both rough and smooth types, similar to those of the meningococcus and the gonococcus.

M. pharyngis siccus was reported by von Lingelshein (1906) to produce acid in glucose, maltose, and fructose, however, the sicca strains of Elser and Huntoon (1909), which closely resembled the organism described by von Lingelshein, fermented sucrose. It was suggested that this difference was due to late acid production which became apparent after the 24-hour incubation period employed by von Lingelshein (Elser and Huntoon, 1909). The production of acid in sucrose was confirmed by Gordon (1921). The systematic position of this organism has been questioned, and it has been suggested that the sicca species is merely a rough variant of one of the naso-pharynx cocci fermenting glucose, maltose, and sucrose (Wilson and Miles, 1946).

Numerous chromogenic, gram-negative cocci have been described as producing a yellow, golden-yellow, or greenish-yellow colony. The descriptions given of the chromogenic cocci are manifold and Wilson (1928) expressed the opinion that the existing confusion is partly due to an omission of adequate cultural descriptions and to different incubation periods for fermentation tests. von Lingelshein's D. pharyngis flavus II agreed culturally with Elser and Huntoon's chromogenic I and II, but only agreed biochemically with their chromogenic II (Elser and Huntoon, 1909). Since some of their strainsof chromogenic group I failed to produce acid in sucrose within 24 hours, Elser and Huntoon (1909) suggested that von Lingelshein's D. pharyngis flavus II possibly belongs to their chromogenic group I. In the same publication Elser and Huntoon reported 4 strains which corresponded culturally and biochemically to von Lingelshein's D. flavus III, but these strains died before observations were completed. From a careful

perusal of the literature, Wilson (1928) stated that it seems clear that colonial appearance, the color of the pigment, and the degree of pigment formation of these organisms are all subject to considerable variation. Notwithstanding the degrees of variation among the chromogenic members, they have been differentiated upon carbohydrate fermentation and pigmentation (Breed et al, 1948). N. flavescens differs from the other chromogenic species in its inability to produce acid from carbohydrates by conventional diagnostic techniques and from the meningococcus in its production of pigment.

Before a consideration of serological investigations on the saprophytic Neisseria, some final comments on colonial characteristics, pigment production, and carbohydrate reactions are made in summary. The conflicting data on pigmentation, colonial structure, and biochemical activity of the gram-negative cocci of the normal nasopharynx has led Wilson and Smith (1928) to conclude that these characteristics are extremely variable and are suitable only for provisional purposes of classification. If species differentiation on carbohydrate fermentation presented in Bergey's Manual (Breed et al., 1948) is to be advocated, Hajek, Pelczar, and Faber (1950) suggested that the establishment of one basal medium is imperative for biochemical studies.

There has been little investigation of the serological relationship of members of the aerobic, non-pathogenic, gram-negative cocci. Wilson and Miles (1946) attributed, as the primary reasons, autoagglutination and lack of stable homogeneous suspensions in saline. D. pharyngis flavus I and II differed somewhat culturally but were easily distinguished by means of agglutination (von Lingelshein, 1906).

Elser and Huntoon (1909) demonstrated that their 3 chromogenic groups were distinguishable from each other by agglutination tests. It was evident from the results of their absorption tests that not all chromogenic, gram-negative cocci placed by them in a group on their cultural and biochemical properties were identical. Complete reciprocal absorption was not always noted. These investigators further reported that none of their chromogenic gram-negative cocci showed any serological relationship to the meningococcus, gonococcus, or N. catarrhalis by agglutination tests. Many strains included in Elser and Huntoon's chromogenic groups showed a tendency to spontaneous sedimentation in saline and in various dilutions of normal serum. This instability was not a constant factor but varied from one generation to the next (Elser and Huntoon, 1909).

In a collection of 60 N. catarrhalis strains, Elser and Huntoon (1909) encountered only 2 strains which formed stable suspensions in saline. Furthermore, these 2 catarrhalis strains did not retain their stability for any considerable period. Martin (1911), Gordon (1921) and Benson et al. (1928) reported similar results. Attempts to form stable, homogeneous suspensions by a variation in salt concentration, and by the addition of alkali or acid were unsuccessful (Gordon, 1921). However, Gordon (1921) reported an agglutinin titer of 1:2000 for an agglutinable strain of catarrhalis. Also, he suggested 3 different types by complement fixation, but the existence of serological groups among the catarrhalis species lacks confirmation.

A serological investigation of N. sicca is entirely lacking. At no time were stable suspensions of this organism obtained in saline by Elser and Huntoon (1909). This finding would be expected in view of the observations of several workers (Gordon, 1921; Wilson, 1928; Martin, 1911) who described N. sicca as a dry, firm colony, difficult to disintegrate, and impossible to emulsify.

Branham (1930) reported N. flavescens to be a serologically homogeneous group and not agglutinated by type antimeningococcal sera. Its ability to produce meningitis under ordinary laboratory conditions appears questionable and therefore was considered as a non-pathogenic species.

A review of the literature reveals some evidence of serological groups among the saprophytic, gram-negative cocci but a serological separation has not been established. N. flavescens has been established as a serologically homogeneous group, however, its serological differentiation from other saprophytic gram-negative cocci has not been studied.

III

EXPERIMENTAL

Cultures. The culture number, species designation, and source of each culture studied is listed in table 1. Although there were some instances where deviations from typical reactions (Breed et al, 1948) were encountered, cultures were given species names according to their fermentation reactions and pigmentation.

Stock cultures were maintained on cystine trypticase agar at 30 C, and were transferred at 3 week intervals. Before a culture was employed in a test, it was transferred daily on trypticase soy agar for at least 1 week, and was maintained thereafter on this medium during the test period.

Cultures of the collection were examined and found to conform to the morphological and cultural description of Neisseria. Colonies of each organism grown on trypticase soy agar were oxidase positive after 48 hours incubation at 35 C (para - aminodimethyl-aniline monohydrochloride reagent). On each culture a microscopic slide agglutination test (Branham, 1945) was performed with polyvalent anti-meningococcic horse serum. Controls of normal horse serum and saline were included with each test. There was no evidence of any serological relationship to the meningococcus.

Carbohydrate fermentation. Strains of the collection were tested for their fermentative capacity on five carbohydrates, namely, glucose, fructose, maltose, sucrose, and mannite. Each carbohydrate

Table 1

Species Designation, Culture Number and Source of Cultures

Species Designation	Culture Number	Source
N. flavescens	157, 155	Dr. S. E. Branham National Institutes of Health
N. flavescens	9746	American Type Culture Collection
N. perflava	2AMS	Army Medical Center
N. perflava	54	State University of Iowa
N. perflava	7925, 12, 24 10-1, 20-1	University of Maryland
N. perflava	51R, 14, 22 28, 8, 17	Johns Hopkins University
N. perflava	1196, 0601 0765	McGill University, Montreal
N. flava	55, 4, 3-1	University of Maryland
N. sicca	1485	Army Medical Center
N. sicca	4-2	University of Maryland
N. sicca	9913	American Type Culture Collection
N. catarrhalis	8193, 8176 7900	American Type Culture Collection
N. catarrhalis	MF1, MF2	Dr. M. Frobisher Communicable Disease Center U.S.P.H., Atlanta
N. catarrhalis	0650, 01197	McGill University, Montreal
N. species	GP5, GP16, F	University of Maryland

was made up as a 10 per cent solution in distilled water, sterilized by Seitz filtration, and then added to previously sterilized cystine trypticase agar to give a final concentration of 0.5 per cent. Results were recorded after 48 hours and again after 7 days incubation at 35 C.

Pigmentation. To determine pigment production, the growth on Loeffler's slants was observed after an incubation of 48 hours and 7 days at 35 C.

Serological Methods. For the preparation of antisera, 18 cultures (protocol 1) were selected from the collection to represent the major groups determined by fermentation reactions and pigmentation. The tube agglutination test was employed to report cross-agglutinations of 55 cultures against the 18 prepared antisera.

1. Preparation of Antisera. Antisera were prepared by intravenous injection of rabbits with living bacterial suspensions. The antigens were saline suspensions of growth washed from a 20- to 24-hour trypticase soy agar slant with 5.0 ml of a 0.85 per cent buffered sodium chloride solution (pH 7.4 with Sörensen's phosphate buffer). The cell suspension, which was prepared on the day of injection, was transferred aseptically to a sterile 10 ml serum bottle, stoppered, and then shaken on a Kahn shaker for 10 minutes. The suspension was then placed in a sterile test tube and diluted with buffered saline to the desired density using a McFarland nephelometer standard for comparison.

Following the schedules outlined in protocol 1, rabbits (in duplicate) were injected with increasing doses of living organisms.

Protocol 1

Injection Schedules for Preparation of Antisera

Time Interval		Intravenous Doses Given on 3 Different Schedules					
		A[1]		B[2]		C[3]	
		Dose	cells/ml*	Dose	cells/ml*	Dose	cells/ml*
1st week	1st day	0.5 ml	300	0.5 ml	600	0.5 ml	900
	2nd day	0.5 ml	300	0.5 ml	600	1.0 ml	900
	3rd day	0.5 ml	600	0.5 ml	900	1.0 ml	900
2nd week	1st day	0.5 ml	600	0.5 ml	900	0.5 ml	1200
	2nd day	0.5 ml	600	0.5 ml	900	1.0 ml	1200
	3rd day	0.5 ml	900	1.0 ml	900	1.5 ml	1200
	4th day					2.0 ml	1200
3rd week	1st day	0.5 ml	900	0.5 ml	1200		
	2nd day	0.5 ml	900	0.5 ml	1200		
	3rd day	1.0 ml	900	1.0 ml	1200		
	4th day	0.5 ml	1200	1.0 ml	1200		
4th week	1st day	0.5 ml	1200	1.0 ml	1200		
	2nd day	0.5 ml	1200	1.5 ml	1200		
	3rd day	1.0 ml	1200	2.0 ml	1200		
	4th day	1.5 ml	1200	2.0 ml	1200		

* Determined with McFarland Nephelometer Standards and expressed as millions.

Organisms used for injection:
[1] 157, 55, 54, 2AMS, 8193, and GP16
[2] 155, 1485, 9913, 7925, 4, and GP16
[3] 12, 24, 4-2, 20-1, 22, 14, 10-1

A trial bleeding was made on the 5th day following the last injection and rabbits with satisfactory titers were exsanguated by cardiac puncture.

Since it was thought that the toxicity of living cells of the non-pathogenic strains might exhibit to some degree the toxic characteristics of the pathogenic Neisseria, schedules A and B were originally followed to prevent loss of animals. Also, it was thought that an extended period of injections with intervals of rest might result in a higher titer antiserum. However, the abbreviated procedure (schedule C) produced similar results and there was no indication of toxic effect to the animals. Occasionally, a higher titer antiserum could be produced by injections for an additional week.

2. Occurrence of Normal Agglutinins. The necessity for normal serum controls in carrying out agglutination tests with this group of organisms (Elser and Huntoon, 1909) led us to investigate the response of some strains in the collection to the action of normal rabbit serum. It was proposed to determine whether normal agglutinins occurred against the aerobic, non-pathogenic Neisseria. The agglutination tests were carried out in the manner described below.

3. Tube Agglutination Technic. The tube agglutination test recommended for the meningococci was followed except for a few minor modifications (Branham, 1945). The procedure employed throughout this study is indicated in detail below. The growth from a 20-to 24-hour trypticase soy agar slant was suspended in 5.0 ml of buffered saline and then shaken for 10 minutes in a Kahn shaking apparatus.

The antigens were prepared by diluting the cell suspension with buffered saline to a density corresponding to a No. 3 McFarland nephelometer standard (900 million cells per ml). Agglutination tests were carried out employing the usual procedure of doubled dilution of antisera (1:20 to 1:10,240), and also the intermediate dilutions of 1:200, 1:400, 1:800, 1:1000, 1:1600, and 1:2000. Doubled dilutions from 1:20 to 1:640 of normal rabbit serum and a saline control were included with each test. Serum dilutions were made with buffered saline. One half ml of antigen was added to an equal quantity of diluted serum contained in Wasserman tubes. The tubes were shaken vigorously, placed in 37 C water bath for 2 hours, then removed and placed at 4 C overnight. On the following day the tubes were allowed to reach room temperature and then read. The results were recorded without shaking the tubes. The titer, was ascertained by the degree of clearing in the supernatant liquid and the amount of agglutinated cells.

4. Agglutinin Absorption Technic. The agglutinin absorption technic was employed to study the serological relationship of certain strains observed to cross-agglutinate. The absorption method used was essentially that described by Stuart, et al.(1940) in the serological identification of coliform bacteria. This procedure is described below. The inoculum for seeding agar plates was a cell suspension prepared by washing the growth from a 20- to 24-hour trypticase soy agar slant with 5.0 ml of buffered saline. Trypticase soy agar plates (20 ml per plate) were seeded with 0.5 ml of the cell suspension, and the inoculum was spread evenly over the agar surface

with a sterile glass rod. The seeded plates were incubated at 35 C for 20 to 24 hours. The growth on each plate was then removed with 5.0 ml of buffered saline and transferred to a sterile centrifuge tube. The cells were sedimented by centrifugation at a speed of approximately 2500 rpm and the saline supernatant decanted and discarded.

To a tube of packed cells, 2.5 ml of a 1:10 dilution of an antiserum was added and mixed. This serum-cell mixture was placed in a 37 C water bath for 1 hour. The tube was removed and the cells sedimented by centrifugation. The serum was transferred to a tube of freshly packed cells, thoroughly mixed and placed in the 37 C water bath for 1 1/2 hours. This procedure was repeated for a 3rd absorption, which was incubated for 2 hours. After centrifugation, the absorbed serum was decanted and used in agglutination tests as a 1:10 dilution. The agglutination technic and results were performed and recorded in the same manner indicated above.

IV

RESULTS

Biochemical and Pigmentation Studies. The results of biochemical and pigmentation studies are given in table 2.

All the strains of Neisseria catarrhalis were comparable in their morphological and biochemical characteristics, but were sufficiently different from other strains of saprophytic Neisseria in their lack of fermentative capacity and absence of pigmentation to constitute a distinct group. The flavescens strains and guinea pig isolates (GP 3, GP 16 and F) demonstrated a lack of biochemical activity which differentiated them from other chromogenic species. These non-fermentative strains were distinguishable from N. catarrhalis by their pigment production. The chromogenic, fermentative strains exhibited degrees of carbohydrate utilization and slight differences in pigmentation. The fermentation of sucrose was considered in this study as a biochemical differentiation between the perflava and flava species. The flava strains were negative in sucrose. The 2 fermentative strains 1486 and 4-2, which gave no evidence of chromogenesis, gave typical carbohydrate reactions for N. sicca (Breed et al., 1948). The variable results observed with strain 9913 did not conform to the biochemical characteristics of N. sicca.

Serological Studies. The results shown in table 3 indicate the extent to which normal agglutinins occurred in 11 normal rabbit sera against 20 strains of aerobic, non-pathogenic Neisseria. The greater

Table 2

Biochemical and Pigmentation Studies on Strains of Aerobic Non-pathogenic Neisseria

Species Designation	Culture Number	Fermentations					Pigmentation
		Glucose	Fructose	Maltose	Sucrose	Mannite	
N. flavescens	157, 155, 9746	-	-	-	-	-	Yellow
N. perflava	2AMS, 54, 12, 24, 31R, 10-1, 22, 20-1, 0765, 0601	+	+	+	+	-	Yellow to caramel
N. perflava	14, 17, 28, 8	-	+	+	+	-	Yellow to caramel
N. perflava	1196	+	+	+	V	-	Yellow
N. perflava	7925	+	+	-	+	-	Yellow
N. flava	55, 3-1, 4	+	+	+	-	-	Yellow
N. sicca	1485, 4-2	+	+	+	+	-	White
N. sicca	9913	V	-	V	V	-	White
N. catarrhalis	8176, 8193, 7900, 0660, 01197, MF1, MF2	-	-	-	-	-	White
N. species	GP3, GP16, F	-	-	-	-	-	Yellow

+, acid; -, no change or alkaline; V, variable.

percentage of strains tested failed to react in normal sera. However, a few strains were agglutinated. Positive reactions were not obtained in serum dilutions greater than 1:40.

The tube agglutination results of 35 aerobic non-pathogenic Neisseria in 16 prepared antisera are given in table 4. An agglutination titer of less than 1:200 was arbitrarily considered as not significant.

As may be seen from table 4, strains of N. flavescens (157, 155, and 9746) formed a distinct serological group. No cross-agglutination between this group and any of the other strains of Neisseria tested was encountered, except for a weak reaction with N. perflava 7925 by the antiserum prepared against strain 155. Strain 155 was agglutinated in a dilution of 1:200 by the antiserum prepared against N. perflava 14; however, strain 9746, which was identical in all other respects to strain 155, demonstrated no cross-agglutination with antiserum 14. The similar antigenic structure of these strains is indicated by the agglutinin absorption experiments presented in table 5.

The guinea pig isolates (GP16, GP3, and F) were comparable in morphological and biochemical characteristics to the flavescens strains, but gave no evidence of serological relationship (table 4). Except for the agglutination of strain GP16 in the antiserum 7925 to a titer of 1:320, the guinea pig strains showed no serological relationship to any of the human strains employed in this study. Rabbits inoculated with GP16 failed to produce a titer of more than 1:200. Furthermore, when other strains of the collection were crossed with antiserum GP16, no antigenic relationship was demonstrated.

Table 3

Agglutinins Occurring in Normal Rabbit Serum for Aerobic Non-pathogenic Neisseria

Antigens	Agglutination Titer with Normal Rabbit Sera										
	#P*	#2	#5	#6	#9	#10	#12	#14	#18	#20	#26
N. flavescens 157	-	-	-	-	-	-	-	-	-	-	-
" 155	-	-	-	-	-	-	-	-	-	-	-
" 9746	-	-	-	-	-	-	-	-	-	-	-
N. perflava 7925	-	-	-	-	-	40	-	-	40	-	-
" 2AM8	20	-	20	-	-	-	-	-	-	-	-
" 54	-	-	20	-	-	-	-	-	-	-	-
" 12	-	-	-	-	20	-	-	-	-	-	40
" 24	-	-	-	-	-	-	-	-	-	-	-
" 14	-	-	-	-	-	-	-	-	-	-	-
" 512	-	-	-	-	-	-	-	-	-	-	-
N. flava 55	-	-	-	-	-	-	-	-	-	-	-
" 4	-	-	-	-	-	-	-	-	-	-	-
" 3-1	-	-	-	-	-	-	-	-	-	-	-
N. sicca 1485	-	-	-	-	-	-	-	-	-	-	-
" 4-2	-	-	-	-	-	-	-	-	-	-	-
" 9913	-	-	-	-	-	-	-	-	-	-	-
N. species GP3	-	-	-	-	-	-	-	-	-	-	-
" GP16	-	-	-	-	-	-	-	-	-	-	-
" F	-	-	-	-	-	-	-	-	-	-	-
N. catarrhalis 8195	Unstable suspension in saline										

* Pooled sera from 6 rabbits

Table 4

Tube Agglutination Reaction of 35 Aerobic, Non-pathogenic *Neisseria* in 16 Prepared Antisera

Antigen		Antiserum															
		157	155	7925	2AM3	54	12	24	20-1	22	14	10-1	55	4	1485	4-2	9913
N. flavescens	157	2560	5120	-	-	-	-	-	-	-	-	-	-	-	-	-	-
"	155	640	5120	-	-	-	-	-	-	-	200	-	-	-	-	-	-
"	9746	640	5120	-	-	-	-	-	-	-	-	-	-	-	-	-	-
N. perflava	7925	-	320	2560	-	-	2560	200	640	400	-	400	320	-	-	-	-
"	2AM3	-	-	320	1600	2560	640	400	1280	640	800	-	-	-	-	-	-
"	54	-	-	-	400	2560	2000	3200	1280	640	-	-	-	-	-	-	-
"	12	-	-	800	400	1280	1000	320	400	200	400	640	800	-	-	-	-
"	24	-	-	-	1600	5120	3200	5120	2560	5120	2560	2560	-	1000	-	-	-
"	20-1	-	-	*	*	*	1000	*	1280	1280	*	*	*	*	-	-	-
"	22	-	-	-	2560	2560	*	2560	1280	1280	1280	*	*	1000	-	-	-
"	14	-	-	200	5120	5120	3200	5120	1280	2560	5120	*	320	*	-	-	-
"	10-1	-	-	-	2560	5120	800	2560	5120	2560	1600	2560	-	1000	-	-	-
"	1196	-	-	-	1000	5120	1000	3200	1000	1280	2560	3200	-	640	-	-	-
"	17	-	-	-	5120	5120	1000	5120	2560	2560	1600	5120	-	3200	-	-	-
"	28	-	-	-	-	320	-	-	-	-	320	-	-	-	-	-	-
"	8	-	-	-	-	800	-	-	-	-	-	-	-	-	-	-	-
"	51R	-	-	-	200	1000	200	*	*	*	*	*	*	*	*	*	*
"	0601	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
"	0765	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
N. flava	55	-	-	-	-	-	-	-	-	-	-	-	1280	-	-	-	-
"	4	-	-	-	-	-	-	-	-	-	-	-	-	5120	-	-	-
"	3-1	-	-	-	-	-	-	-	-	-	-	-	200	-	-	-	-
N. sicca	1485	-	-	-	-	-	-	-	-	-	-	-	-	-	2000	640	-
"	4-2	-	-	-	-	-	-	-	-	-	-	-	-	-	1280	2560	-
"	9913	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	3200
N. spp.	GP3	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
"	GP16	-	-	320	-	-	-	-	-	-	-	-	-	-	-	-	-
"	F	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
N. catarrhalis	8176																
	8193, 7900																
	0650, 01197	All catarrhalis strains exhibited an instability in saline.															
	MF1, MF2																

* Unstable suspension in saline.

Table 5

Absorption of N. flavescens Antisera with N. flavescens Strains

Antiserum		Organism Used for Absorption	Agglutination Titer with N. flavescens strains	
Number	Treatment		157	155
157	Unabsorbed		2560	640
	Absorbed	157	-	-
		155	2560	-
155	Unabsorbed		1600	5120
	Absorbed	155	-	-
		157	-	1280

The perflava strains, which have been characterized as fermenting all or nearly all of the 5 carbohydrates and producing a pigment, constituted another serological group. Cross-reactions among perflava strains were easily demonstrated, but the flava strains and other fermentative strains, i.e. N. sicca, demonstrated no agglutinin titers in perflava antisera. As may be seen from table 2, the biochemical characteristics of the perflava members suggested at least two groups; one group which fermented all carbohydrates except mannite, and a second group which differed only in the fermentation of glucose. Two strains (14 and 17) of the less fermentative perflava organisms were found to be closely related serologically to the more typical perflava strains which fermented all carbohydrates. Conversely, strains 8 and 28 presented little serological evidence of similarity to the other perflava members of the collection. By the same standards, strains 8 and 28 are wholly unrelated to other species tested in this study. It was noted with a great deal of interest that perflava strain 7925, which does not ferment maltose, demonstrated degrees of agglutination in some prepared perflava antisera (12, 24, 20-1, 22, and 10-1), but that the perflava strains demonstrated only slight agglutinability in antiserum prepared against 7925.

As previously encountered by Elser and Huntoon (1909), it also was found that certain chromogenic strains, particularly 20-1 and 22, varied in their ability to form stable suspensions in saline. This observation is indicated in table 4. The organisms were used as antigens on 16 consecutive days. Each day, the two strains were cross-agglutinated against a single antiserum beginning with

antiserum 157 on the 1st day and ending with antiserum 9913 on the 16th day. Therefore, it is not to be construed that cross-reactions with other perflava antisera could not occur if stable suspensions could be formed in saline. Strains 0601 and 0765 were continually rough and strain 31R became rough during this study and remained so.

The close antigenic relationship of some of the perflava strains, as determined by cross-absorption tests, is summarized in table 6.

Insufficient data was obtained to indicate a serological relationship among those pigmented strains which fermented some of the carbohydrates but which failed to ferment sucrose, i.e. N. flava. The cross-agglutination which various strains of N. perflava exhibited in antisera prepared against the flava strains 55 and 4 (table 4) is evidence of some serological relationship of perflava strains to these flava strains. The flava strains were sufficiently different to be serologically separated from the flaveseens and perflava groups.

The 2 strains of N. sicca, 1485 and 4-2, which fermented all carbohydrates and gave no evidence of pigmentation, presented serological evidence for the possible establishment of another group. As shown in table 4, strains 1485 and 4-2 cross-agglutinated; in addition, complete reciprocal absorption resulted between the two strains. Table 7 shows the results of this absorption experiment. The extreme variability of strain 9913, labeled N. sicca, in its fermentative capacity (table 2) would appear to preclude any serological relationship with either the sicca group or the chromogenic groups. The antiserum 9913 did not agglutinate any of the other Neisseria strains tested. Furthermore, none of the other prepared antisera agglutinated strain 9913.

Table 6

Absorption of *N. perflava* Antisera with *N. perflava* Strains

Antiserum		Organism Used For Absorption	Agglutination Titer with *N. perflava* Strains									
Number	Treatment		2AMS	54	24	12	10-1	7925	1196	17	14	22
2AMS	Unabsorbed		1600	400	1600	400	2560	-	1000	5120	5120	2560
	Absorbed	2AMS	-	-	-	-	-	-	-	-	*	*
		54	160	-	-	160	-	-	-	-	*	*
54	Unabsorbed		2560	2560	5120	1280	5120	-	5120	5120	5120	2560
	Absorbed	54	-	-	1280	160	-	-	1280	-	*	*
		24	-	640	-	-	-	-	-	-	*	*
		10-1	160	1280	2560	640	-	-	2560	1280	*	*
		1196	-	320	-	-	-	-	-	-	*	*
24	Unabsorbed		400	3200	5120	320	2560	200	3200	5120	5120	2560
	Absorbed	24	-	-	-	-	-	-	-	-	*	*
12	Unabsorbed		640	2000	3200	1000	800	2560	1000	1000	3200	*
	Absorbed	12	-	-	-	-	-	-	160	-	*	*
10-1	Unabsorbed		-	-	2560	640	2560	400	3200	5120	*	*
	Absorbed	10-1	-	-	-	640	1280	-	320	640	*	*
7925	Unabsorbed		320	-	-	800	-	2560	-	-	200	-
	Absorbed	7925	-	-	-	-	-	160	-	-	*	*

*Unstable suspension in saline.

Table 7

Absorption of *N. sicca* Antisera with *N. sicca* Strains

Antiserum		Organism Used For Absorption	Agglutination Titer with *N. sicca* Strains	
Number	Treatment		1485	4-2
1485	Unabsorbed		2000	1280
	Absorbed	1485	-	-
		4-2	-	-
4-2	Unabsorbed		640	2560
	Absorbed	4-2	-	-
		1485	-	-

All of the strains of N. catarrhalis were observed to spontaneously agglutinate in buffered saline, and therefore could not be studied by the agglutination procedure described herein. Nevertheless, rabbits were injected with the N. catarrhalis strain 8193. This prepared antiserum failed to cross-agglutinate with any of the other strains of Neisseria tested.

V

DISCUSSION

The results presented indicate that some of the species of aerobic, non-pathogenic Neisseria as classified in Bergey's Manual (Breed et al., 1948) are serologically related. This does not imply that all strains of a given species which we have studied are serologically identical. Neither cross-agglutination in an antiserum to the strength of its homologous strain nor complete reciprocal absorption was always achieved with strains considered to be equivalent in biochemical and serological characteristics.

This investigation has considered representative strains of each species with the exception of N. subflava, a species which was not isolated at the University of Maryland and was not available in the collections known to us. The serological differences together with biochemical and pigmentation characteristics suggest certain groups among the aerobic, saprophytic Neisseria. The non-chromogenic strains fall into two groups: (1) N. catarrhalis and (2) N. sicca. The chromogenic strains comprise at least three groups: (1) fermentative strains, (2) N. flavescens, and (3) guinea pig isolates of Pelczar, Hajek, and Faber (1949).

A strict correlation between serological and biochemical groups observed with the fermentative chromogenic strains was not always apparent. The cross-agglutinations (table 4) observed with the typical perflava strains (2AMS, 54, 12, 24, 10-1, and 22) and their antisera represent serological data for the establishment of a

specific group. Strains 14 and 17 were negative in glucose, but their cross-reactions in perflava antisera (2AMS, 54, 12, 24, 20-1, and 22) resulted in evidence indicative of a close relationship to the typical perflava strains. The glucose negative strains 8 and 28 showed little serological relationship to perflava group; however, these strains did not cross with antisera of other groups of aerobic, non-pathogenic Neisseria, i.e., flavescens, sicca, or flava. The strain 7925 was negative in maltose, failed to agglutinate in perflava antisera 2AMS, 54, and 14, but cross-agglutinated in perflava antisera 12, 24, 20-1, and 22. The flava strains 55, 4, and 3-1, which were negative in sucrose, demonstrated a complete absence of cross-agglutination in perflava antisera. However, a relationship of certain perflava and flava strains was evidenced by the cross-agglutination of strains 7925, 12, 24, 22, 10-1, 1196, and 17 in flava antisera. Thus the implications are that some serological relationship exists among the fermentative chromogenic species. The sucrose-negative strains (N. flava) showed none of the serological specificity of the perflava group, but other fermentative strains, which fail to ferment glucose or maltose, appeared to fit the serological pattern of the perflava group. These results do not preclude the serological relationship of some perflava strains to flava strains as shown in table 4. These groups were sufficiently different as to be distinguished by agglutination tests, but demonstrated some degree of serological relationship.

A serological subgroup of the perflava group was suggested by Elser and Huntoon (1909). The minor cross-agglutination of strains

8 and 28 in perflava antisera (table 4) are suggestive of such a subgroup, but the value of a subgroup would be questionable from the results presented. Any serological subgroup of the perflava group must await the establishment of additional isolates which vary sufficiently from the perflava group concluded in this study.

The non-fermentative N. flavescens strains, 157, 155, and 9746, formed a serologically homogeneous group and showed no relationship to the perflava or flava groups, or to the guinea pig isolates. Differences in pigments of flavescens strains and guinea pig isolates have been noted (Branham, 1951). The designation of a new species, Neisseria caviae, for the guinea pig isolates (Pelczar, 1951) is substantiated by their complete absence of serological relationship to the flavescens, perflava, or flava groups and by their inability to ferment carbohydrates. N. caviae and N. flavescens are easily distinguished from N. catarrhalis by their chromogenesis.

Although no serological data was obtained in this study on N. catarrhalis, its characteristics of no pigment and biochemical inactivity towards carbohydrates are sufficient points of differentiation . Two other non-chromogenic strains which we studied, i.e. N. sicca 1485 and 4-2, gave positive reactions in all 4 of the carbohydrates and were serologically homogeneous organisms. N. sicca is characterized in Bergey's Manual (Breed et al., 1948) as precipitating spontaneously when suspended in saline. In our studies the sicca cultures 1485 and 4-2 were smooth non-chromogenic strains, easily suspended in saline, and conformed to the biochemical activity described for N. sicca. The sicca strain 9913 was extremely variable in its carbohydrate reactions but in other respects was a suitable

organism for serological study. This culture failed to show any serological relationship to any other strains studied.

No adequate explanation can be offered for the reported observation that various aerobic saprophytic Neisseria spontaneously agglutinated in saline. The instability of these organisms in saline is exemplified by the inconstant, spontaneous agglutination of strains 20-1, 22, and 14. The growth of strains 0601 and 0765 was rough, adhered to agar surface, and was removed with great difficulty. Spontaneous agglutination may be due to a form of roughness, but the rough growth of strains 0601 and 0765 is not comparable to the phenomenon associated with the spontaneous sedimentation of an organism which appears to suspend in saline, spontaneously agglutinates and then settles out. All strains of N. catarrhalis which we studied exhibited an instability in saline. This phenomenon was not effected by the lowering of sodium chloride concentration from 0.85 per cent, or by a variation in hydrogen ion concentration.

Normal agglutinins against the aerobic, non-pathogenic Neisseria were not demonstrated in the sera studied. Rarely was spontaneous agglutination observed in normal rabbit sera without a sensitivity in saline.

As previously indicated, aerobic saprophytic Neisseria are primarily differentiated on a basis of chromogenesis and carbohydrate fermentation. However, the difficulties which arise in the interpretation of Neisseria pigments and the variations in carbohydrate fermentation which may be influenced by the composition of the basal medium necessitates consideration of other characteristics in the classification of the Neisseria. The results of this serological study offer an

additional means of differentiation, which in general can be correlated with chromogenesis and carbohydrate fermentation.

The present observations have not altered the general agreement that N. catarrhalis, which is non-chromogenic, and biochemically inactive by usual diagnostic procedures, is a valid species. Two strains, 1485 and 4-2 (N. sicca), were non-chromogenic, fermented the carbohydrates glucose, fructose, maltose, and sucrose, and were serologically related. Complete reciprocal absorption resulted between the 2 strains. Thus from the results obtained with these 2 strains, N. sicca would appear to be a distinct species.

Until the isolation of N. caviae, the systematic position of N. flavescens was firmly established on chromogenesis and lack of carbohydrate fermentation. It has been shown to be a distinct serological group, and to be unrelated to other aerobic saprophytic Neisseria. Although differences in pigments of these non-fermentative species was previously indicated (Branham, 1951), the lack of serological relationship to N. flavescens was the more reliable means of differentiation of N. caviae.

In view of the serological data presented, the validity of 3 species (N. perflava, N. flava, and N. subflava) among the fermentative chromogenic strains of aerobic saprophytic Neisseria becomes questionable. Only fermentative chromogenic strains which were sucrose negative (N. flava) deviated sufficiently from the serological pattern of the perflava group as to be distinguished by agglutination tests. However, a serological relationship of perflava strains to flava strains has been indicated. It is suggested, therefore, that the name Neisseria pharyngis be adopted for the fermentative chromogenic strains of

aerobic non-pathogenic Neisseria. This suggestion is in line with the opinion expressed by the British investigators. The perflava and flava groups described in this study could be assigned as varieties of N. pharyngis.

VI

SUMMARY AND CONCLUSIONS

An investigation of 55 strains of aerobic, non-pathogenic Neisseria was undertaken to determine possible serological relationships which would aid in their classification. The cross-agglutination reactions of these strains in 18 antisera, produced by intravenous injection of rabbits, were correlated with results of studies on carbohydrate fermentation and pigment production, previously recorded. Agglutinin absorption tests were employed to establish further the relationship of serological groups observed from cross-agglutination results.

The perflava strains, which were pigmented and fermented all or nearly all of the recommended carbohydrates, formed a serological group. The similar antigenic relationship of the perflava strains was demonstrated by cross-absorption tests. The chromogenic flava strains, which fermented some of the carbohydrates but failed to ferment sucrose, deviated sufficiently from the serological pattern of the perflava group as to be distinguished by agglutination tests. However, a serological relationship of perflava strains to flava strains was noted.

Branham's strains of N. flavescens were serologically homogeneous. The non-fermentative, chromogenic, guinea pig isolates, designated as N. cavise, were not related serologically to N. flavescens or to any of the other species studied.

All strains of N. catarrhalis were non-chromogenic, biochemically inactive towards carbohydrates, and formed unstable suspensions in saline.

Two non-chromogenic strains, which fermented all recommended carbohydrates, were serologically homogeneous organisms, and conformed in general to the description of N. sicca. Complete reciprocal absorption resulted between the two strains.

The name Neisseria pharyngis was suggested for the fermentative chromogenic strains of aerobic non-pathogenic Neisseria.

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