SEROLOGICAL CHARACTERISTICS OF COAGULASE POSITIVE AND NEGATIVE STAPHYLOCOCCI

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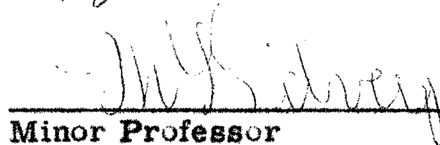
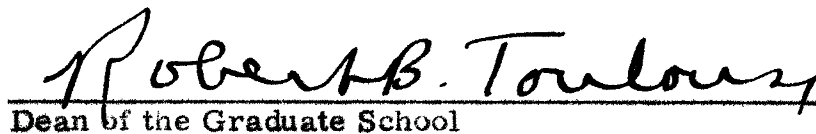


Minor Professor

Director of the Department of Biology



Dean of the Graduate School

SEROLOGICAL CHARACTERISTICS OF COAGULASE POSITIVE AND NEGATIVE STAPHYLOCOCCI

THESIS

Presented to the Graduate Council of the
North Texas State University in Partial
Fulfillment of the Requirements

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By

John H. Newton, B. A.
Denton, Texas

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CHAPTER I

INTRODUCTION

Most bacteriologists recognize that the pathogenicity of staphylococci may be indicated by the coagulase activity exhibited. This is perhaps the most reliable indication of pathogenicity, in *Staphylococcus* species or strains. The enzyme coagulase has the ability to clot plasma of human and rabbit blood. It will also clot plasma of other species of animals, but the amount of clotting will vary. Since the emergence of antibiotic-resistant organisms, the need has arisen for a rapid but still accurate means of identifying or differentiating the strains of potentially pathogenic staphylococci.

In the past, bacteriophages have been used to type the staphylococci. The bacteriophage has a specific affinity for certain strains of bacteria. However, the susceptibility of a strain of *Staphylococcus* to a phage will vary. Live and Nichols in 1965 stated that a large number of strains of staphylococci were not typable by phage methods (6). They also showed that a large majority of staphylococcal strains, persistently untypable with phages, could be typed by serological methods. Normally, a positive reaction involving a bacteriophage specific for a bacterium will show lysis of the bacterium.

Other techniques have been devised which may prove to be useful. In 1952, Oeding, following an epidemic of mastitis, using agglutinin absorption, obtained a serum containing antibody specific for the epidemic strain. He produced rabbit immune sera using formalin-killed bacteria, which he also used for absorption. Then, he was able to determine the pathogenic strain using the standard slide agglutination test. In this work, Oeding, also found evidence for blocking antigens. It was thought that one antigen might block agglutination of other antigens presumably situated deeper in the cell. He discovered that certain antigens were perhaps blocked in 18-hour old cultures, but were not blocked in autoclaved dead cultures (7). Since then, the method of slide agglutination has been revised and extended by Oeding 1953 (8), Haukenes and Oeding 1960 (4), and Hofstad 1961 (5). These recent investigations have shown that in certain cases an antigen may be discovered only after autoclaving. At present, there are procedures available for the preparation of about 20 different antisera for the typing of staphylococci. These methods vary somewhat so that the results when compiled show some discrepancies. Therefore, the usefulness of these would be questionable.

In 1941, Coons _et al._ (2) developed the technique of immunofluorescence. He was able to tag immune serum with a fluorescent group. Such a fluorescent tag will show fluorescence if it absorbs light energy of one frequency and emits light of another frequency.

Using this, he was able to describe the immunological properties of an antibody protein containing a fluorescent group. The technique was later improved and extended by Coons and Kaplan in 1950. They improved the methods for detection of antigens and showed the localization of some antigens in tissue cells (3). Thus the fluorescent antibody technique has become a specialized serological procedure which consists of an antibody-antigen complex made visible by a fluorescent dye incorporated into the system, usually tagged onto the antibody protein. This has lead to many variations and situations where the immunofluorescent technique has been employed to determine the identity of unknown antigens.

The work involving staphylococci with immunofluorescence was initiated by Cohen and Oeding in 1962, at which time they reported on the comparison of serotyping of fluorescent antibodies and slide agglutination. These workers showed almost complete correlation between the two serological tests. In addition, one fluorescent antibody reagent showed a somewhat broader spectrum of activity than a corresponding agglutination serum (1).

Oeding 1960 (10), in his review article discusses the immense difficulties and problems encountered when studying the antigenic properties of the staphylococci. Some of these are lack of type specific antigens, many heat labile antigens, blocking agglutinins, quantitative variation of the staphylococcal antigens from one strain

to another, and varying ability of antigens to mediate agglutination and to produce antibodies. Methods which are used successfully on related bacteria fail when employed for the grouping of staphylococci. Oeding feels that since the antigenic structure of this organism is so complex, many workers have contented themselves with the morphological and biochemical behavior rather than the serological and immunological characteristics (10).

In the past, the staphylococci have been characterized by various means. Some of these have been tedious and time consuming, with the majority of these characteristics being obtained from morphological and biochemical data. This data, however, cannot differentiate between strains of bacteria. Serologically related methods may if properly employed give some reliable grouping of the staphylococci. Some of these methods may prove to be more useful than others. For this work, the decision was made to contrast two serological tests, that of the tube agglutination technique and the fluorescent antibody technique for correlation with the coagulase and other characteristics of *Staphylococcus* strains. This has been a preliminary survey in the hope that as further knowledge is obtained about the staphylococci, grouping of the organisms will become more routine and relatively less complex.

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CHAPTER II

MATERIALS AND METHODS

Bacterial Strains

The forty-two different strains of staphylococci used in this study were collected from two sources, milk and the stock cultures of North Texas State University. The stock cultures are numbered as follows: 116-Mc, 117-NTSU 18, 118-NTSU 20, 119-ATCC 6538 p, 120-ATCC 4776, and 122-Mc.

Culture Media

The individual cultures were streaked on Trypticase Soy Agar (Baltimore Biological Laboratory) (1) plates and examined at twenty-four hours at 37°C for contamination. A smear was gram stained from each culture to determine purity of culture and morphology; transfers were then made to trypticase soy agar slants for working cultures. These were inoculated to Mannitol Salt Agar and *Staphylococcus* 110 Agar (Difco) (4). Both of these media contain a percentage of NaCl (7.5%), which is highly selective for staphylococci. After incubation for forty-eight hours at 27°C, pigmentation on each medium was noted.

Hemolytic Activity

The hemolytic activity of staphylococci strains was checked by inoculating to blood agar (trypticase soy agar + five per cent sterile citrated rabbit blood) incubated at 37°C and read for hemolysis at forty-eight hours growth.

Coagulase Test

The enzyme coagulase was tested for by the standard coagulase test (Difco) (4). Blood plasma (0.5 ml) was added to a trypticase soy broth culture (0.5 ml) of each organism. The mixture was incubated at 37°C for two hours. After the second hour, the tubes were visually checked for coagulated plasma. Any coagulation was considered a positive test.

Antigen Preparation

All antigens and vaccines were prepared by inoculating a trypticase soy agar slant (Baltimore Biological Laboratory) in a 500 ml screw top bottle with the appropriate organism. The culture was allowed to incubate for forty-eight hours. Harvesting was accomplished by washing the slants with twenty milliliters physiological saline buffered to pH 7.8 with Sorenson's Phosphate Buffer (7). The suspended cells were placed in standard petri dishes under ultraviolet light for ten minutes. Following irradiation sterility of vaccines was checked by inoculation into Fluid Thioglycolate Medium (Difco) (4) and results

read after twenty-four hours incubation. The non-viable suspensions were then divided into two aliquots. One half of each suspension was placed into 200 ml sterile screw top bottles and diluted to 3 x 10^8 cells per ml with buffered saline pH 7.8 (Sorenson's Phosphate Buffer). This dilution was used for the standard tube agglutination test. The remainder of each suspension (10 ml) was diluted as above to 10^9 bacterial cells per milliliter for injection into rabbits.

Antiserum Production

Immune serum was produced in rabbits from the two groups of cultures listed in Table I.

TABLE I

ANTIGEN GROUPS

Group I		Group II	
	(from NTSU stock cultures)		(from milk isolates)
#118	NTSU 20	#14	coagulase (-)
#119	ATCC 6538 p	#33	coagulase (+)
#120	ATCC 4776	#36	coagulase (+)
#122	Mc	#42	coagulase (+)

A sterile two milliliter syringe and a twenty-six gauge needle were used to make the injections into the marginal ear vein of the rabbit.

TABLE II

INJECTION SCHEDULE

Day of Schedule	Amount of dilute vaccine in ml.
1	0.5
3	0.5
5	1.0
7	1.0
9	2.0
11	2.0
13	2.0
15	3.0

The schedule of injections is shown in Table II.

After the final injection, a week was allowed to elapse before bleeding. Bleeding was done with a standard ten milliliter syringe and twenty gauge needle by intra-cardial puncture. A clot was allowed to form, which was then removed and the remaining serum was centrifuged at 2,000 revolutions per minute for twenty minutes. Serum was drawn off, using a capillary pipette, and placed into labeled sterile (200 ml) small screw top bottles for storage at 4°C.

Agglutination Tests

The titer of each antiserum was checked by the tube agglutination method of Edwards and Ewing (1962) (3). This consisted of preparing twofold serial dilutions of antiserum in buffered saline (pH 7.8), to which was added 0.5 ml of bacterial antigen. The mixture was shaken and incubated overnight in the refrigerator. Agglutination was read at twenty-four hours, with any visible clumping of cells considered a positive test. A cell control was included.

Each staphylococcal strain was tested by cross agglutination against each of Group I antisera. The strains which were not grouped by reaction with Group I antisera were then reacted with Group II antisera.

Fluorescent Techniques

The fluorescent equipment used for this study was as follows: HBO 200 Super Pressure Mercury Lamp with special Zeiss housing, transformer and combination choke-igniter, filters (heat filter, BG-12 filter, and UG-5 filter), and Bausch and Lomb microscope. This microscope was fitted with a 97x oil immersion objective containing an iris diaphragm. A Bausch and Lomb Cardioid condenser was used to provide the dark field. Cargille's oil was used for better illumination and resolution and was placed on the condenser.

The Fluorescent techniques employed were the direct and the indirect procedures, according to the Difco Manual of Fluorescent

Antibody Techniques (6). The labeled anti-gamma globulin was obtained from Immunology, Inc., Glen Ellyn, Illinois, in a dilution of 1:5. The labeling agent was fluorescein isothiocyanate. The conjugation of serum protein number 120 with the fluorescein isothiocyanate was accomplished using the method of the Baltimore Biological Laboratory bulletin of Fluorescent Antibody Conjugation and Antigen "Staining" Technique (5).

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CHAPTER III

RESULTS AND DISCUSSION

The results of pigmentation on Staphylococcus 110 Agar are shown in Table III. The table was constructed to compare pigmentation results to coagulase reactions. The results shown in Table III indicate that ten subgroups were possible using Staphylococcus 110 Agar. Each group with the exception of number five was subdivided into two subgroups. As is seen in the table only one pigmentation showed a clearly defined coagulase activity. This was Group V, showing yellow pigmentation with negative coagulase tests. Each of the other pigmentations showed both coagulase activities. The majority of ivory and yellow orange pigmented strains showed positive coagulase reactions. The brown yellow and light yellow, however, seemed to be evident in both coagulase positive and negative strains.

The results from Mannitol Salt Agar are shown in Table IV. Here again, as in Table III, there were five major groups, differentiated as to coagulase reactions and pigmentation results. However, in this case only three of these groups showed subgrouping. The yellow pigmented strains seemed to be predominantly coagulase positive. The coagulase negative strains showed white and orange pigmentations,

TABLE III

PIGMENTATION ON STAPHYLOCOCCUS 110 AGAR

Positive coagulase			Negative coagulase		
Group	Strain No.	Pigmentation		Strain No.	Pigmentation
I. A.	2 23 29 30	ivory	B.	10 17	ivory
II. A.	3 7 18 21 33 38	brown yellow	B.	27 31 35 40 41	brown yellow
III. A.	4 5 6 8 16 26 28 32 36 42	light yellow	B.	9 15 19 20 34 37 39 43	light yellow
IV. A.	12 13 22 24	yellow orange	B.	11	yellow orange
V.				14 25	yellow

TABLE IV

PIGMENTATION ON MANNITOL SALT AGAR

Positive coagulase			Negative coagulase		
Group	Strain No.	Pigmentation		Strain No.	Pigmentation
I. A.	2	yellow	B.	35	yellow
	3			40	
	4			41	
	5			43	
	6				
	7				
	8				
	28				
	29				
	30				
	32				
	38				
	42				
II. A.	12	white	B.	9	white
	13	yellow		10	yellow
	16			11	
	18			14	
	21			15	
	22			17	
	23			19	
	24				
III. A.	33	yellow	B.	34	yellow
	36	orange		37	orange
	26			39	
IV.				25	white
				20	
V.				27	orange
				31	

while the yellow pigmented were largely coagulase positive. The white-yellow and yellow-orange each showed almost equal numbers of coagulase positive and coagulase negative strains.

No more than two or three strains were grouped together in results from comparison of the two media used. The pigments did not correlate, the placement of the numbered strains did not correlate, and the number of subgroups did not correlate. However, in the results shown, it was noted that a more clearly defined coagulase reaction could be ascertained when solid or pure colors were shown. The intermediate pigments showed a mixture of coagulase activities. In this regard, it would be suggested that for differentiation between coagulase positive or pathogenic staphylococci and coagulase negative strains, the pigmentation results should be used only in conjunction with other tests.

Another test performed in addition to the pigmentation and coagulase tests was the hemolytic test. This test was performed on blood agar and the results are given in Table V. Most strains were beta hemolytic. By far, the greatest number of beta hemolytic strains were coagulase positive. There were nine strains which were beta hemolytic and coagulase negative. In addition, six strains were found to be alpha hemolytic and coagulase negative.

The agglutination reaction results are shown in Tables VI and VII. These have been classed according to antiserum groups, to individual coagulase activities, and to cross reaction titers. Table VI

TABLE V

HEMOLYSIS AND COAGULASE

Positive coagulase			Negative coagulase	
Group	Strain No.	Hemolysis	Strain No.	Hemolysis
I.	2	Beta	9	Beta
	3		11	
	4		14	
	5		20	
	6		25	
	7		27	
	8		41	
	12		43	
	13			
	16			
	18			
	21			
	22			
	23			
	24			
	26			
	28			
	29			
	30			
	32			
	33			
	36			
	38			
	42			
II.			10	Alpha
			19	
			31	
			34	
			39	
			40	

TABLE VI

GROUP I ANTISERA

Class		Strain No.	Coagulase Reaction	Antisera 118	119	120	122
I.	A.	23	+	1:40	1:160	1:40	1:80
		2		1:20	1:40	1:160	1:160
		7		1:40	1:20	1:160	1:80
		18		1:20	1:40	1:20	1:20
		28		1:80	1:80	1:160	1:20
		29		1:20	1:20	1:20	1:80
		12		1:40	1:20	1:160	1:20
	B.	25	-	1:80	1:160	1:160	1:40
		19		1:20	1:40	1:40	1:40
		31		1:20	1:80	1:320	1:40
II.	A.	4	+	1:40	-	1:160	1:40
		13		1:20	-	1:20	1:40
		21		1:80	-	1:20	1:20
	B.	35	-	1:40	-	1:40	1:40
III.		26	+	-	-	1:20	1:320
		6		-	-	1:320	1:40
		38		-	-	1:1280	1:40
IV.		9	-	-	-	-	1:20
		11		-	-	-	1:20
		41		-	-	-	1:20

refers to Group I antisera (numbered 118, 119, 120 and 126). This group of antisera was cross reacted with all of the strains (numbered 2 through 43). The resulting agglutination titers were then arranged according to the degree of cross reaction. This yielded an arrangement of four groups. Each antiserum did not react with every strain tested. Some strains showed cross reaction titers with all antisera while others did not. These varying cross reaction titers lead to the classes found in Table VI. Class I was by far the largest class, containing cross reaction titers for each of the four antisera. Class I was further divided into coagulase positive and coagulase negative portions. Class II also contained similar cross reaction titers between coagulase positive and negative and was so subdivided. Class III and Class IV were not subdivided. There remained a number of strains which showed little or no cross reaction with Group I antisera. Four additional strains (numbered 33, 36, 14, and 42) were chosen from these strains to produce additional antisera because of their degree of reaction with Group I antisera. These latter four were called Group II antisera.

Table VII shows the agglutination results obtained from cross reacting strains not classed by Group I antisera but classed with Group II. The results were arranged as with Group I. Four different arrangements of cross reacting titers were obtained and these were divided into subclasses according to coagulase positivity or negativity. Class II was found to be the largest group. This class showed cross

TABLE VII

GROUP II. ANTISERA

Class		Strain No.	Coagulase Reaction	Antisera 33	36	14	42
I.	A.	30	+	1:20	1:80	1:40	1:20
		36		1:20	1:320	1:20	1:20
		8		1:80	1:40	1:40	1:20
	B.	35	-	1:80	1:20	1:20	1:40
II.	A.	32	+	1:20	-	1:20	1:40
		4		1:20	-	1:80	1:160
		21		1:40	-	1:20	1:20
		24		1:20	-	1:80	1:20
		42		1:160	-	1:160	1:80
		3		1:160	-	1:40	1:80
	B.	39	-	1:20	-	1:20	1:20
		27		1:20	-	1:20	1:80
III.		13	+	-	-	1:160	1:20
		16		-	-	1:20	1:80
		5		-	-	1:20	1:40
IV.	A.	17	-	1:20	-	-	1:40
		37		1:20	-	-	1:80
		34		1:20	-	-	1:20
	B.	43	-	-	1:20	1:40	-
		10		-	1:80	1:160	-
		14		-	1:20	1:160	-
		15		-	1:20	1:20	-
V.	(Groups not classified by these reactions)						
	A.	40	-	1:20	-	-	-
	B.	20	-	-	1:40	-	-
	C.	22	+	-	-	-	1:40
	D.	33	+	1:160	-	-	-

reactions with all antisera except number 36. Class I showed cross reaction titers with all antisera. In Class IV, A and B were placed together because of the lack of titers from two antisera. Odd cross reacting titer results were placed into Class V. These could not be classed elsewhere in either Group I or Group II antisera.

Staphylococcal strains numbered 4, 13, and 21 showed interesting results when reacted with Group II antisera. Numbers 4 and 21 were classed together in both groups of antisera. This perhaps would suggest similar antigenic nature or at least the presence of certain like antigenic properties. Number 13 was classed with two other strains in Table VII showing no cross reaction titers from antisera numbered 33 and 36.

Results shown in Tables VI and VII suggest that eight major agglutination types of staphylococci with similar antigenic patterns were established in this survey. This substantiates the work of earlier investigators. The exact number of types will vary due to differences in methods, techniques used, and strains of staphylococci used. Cowan, using the slide agglutination method, classified strains of staphylococci into three main types (2). Christie and Keogh (1) added six and Hobbs (3) found four more types to bring the total to thirteen. Still other workers reduced the number of patterns or types. Oeding and Williams found a large number of antigenic patterns; however it was later found that four of the patterns accounted for fifty per cent

of the strains (6).

The results of the indirect fluorescent antibody test using the fluorescein isothiocyanate rabbit anti-gamma globulin proved inconclusive, as all tests of each strain and antiserum showed positive fluorescence. This may be explained by Oeding, who stated that it has been repeatedly observed that non-sensitized rabbits may contain circulating antibodies that react with certain staphylococci (4). If these "normal" antibodies were present in the rabbit serum, then the results from the indirect fluorescent antibody test were meaningless as far as any differentiation was concerned. A normal serum control was run, along with an indirect test involving Eschericia coli and Streptomyces griseus. Each showed no fluorescence.

The direct fluorescent antibody test employed fluorescein tagged immune serum. Results are shown in Table VIII. Group I shows strains which gave positive fluorescence, and is divided into classes according to coagulase reactions. The immune serum used in this test was number 120.

For Group I antisera, with the exception of number 28, which was fluorescent negative, all of agglutination Class I and Class II coagulase positive strains were found to be in fluorescent positive Group I. Agglutination Class I and Class IV coagulase negative strains were shown to be in fluorescent negative Group II. The additional coagulase positive fluorescent negative strains were also difficult to group by

TABLE VIII

DIRECT FLUORESCENT ANTIBODY TEST RESULTS

Group	Class	Positive coagulase Strain No.	Class	Negative coagulase Strain No.
I. Fluorescence	A.	2	B.	10
		3		15
		4		17
		5		19
		6		20
		7		25
		8		26
		12		31
		13		35
		16		37
		18		39
		21		43
		23		
		24		
		29		
		32		
		33		
		38		
		42		
II. No Fluorescence	A.	22	B.	9
		28		11
		30		14
		36		27
				34
				40
				41

agglutination tests.

For Group II antisera, agglutination Class II and Class III coagulase positive strains were shown to be in the Group I of the positive fluorescent results.

While very few of the coagulase positive did not show fluorescence, a large number of false positives and a small number of false negatives were shown by the fluorescent antibody technique. It appears from these results that the agglutination test would be more reliable as a means of detection.

Some correlation was obtained between the agglutination methods and the fluorescent techniques. Most of the strains grouped by the serological agglutination reactions were placed in common groups by the fluorescent method. This would indicate that the fluorescent method has a higher degree of sensitivity but less specificity.

Only one antiserum was used for the fluorescent results. If more antisera were developed and employed for the fluorescent testing after proper absorption procedures, then the high degree of sensitivity put forth by this test should enable a worker to rapidly but still accurately determine the antigenic grouping of a strain of staphylococci, and could lead to a rapid method for determining the pathogenicity of a staphylococcal strain. Without extensive antigenic analysis, however, it is apparent that these serological procedures alone cannot be used to rapidly detect coagulase positive "pathogenic" staphylococci.

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CHAPTER IV

SUMMARY

The results of the pigmentation on Staphylococcus 110 Agar and Mannitol Salt Agar vary considerably. The comparison of these media showed no more than two or three strains of staphylococci grouped together. Neither the pigments, placement of numbered strains, nor number of subgroups correlated. However, when solid or pure colors were shown, a more uniform coagulase reaction was evident. Reliable differentiation between coagulase positive and coagulase negative strains of staphylococci may be obtained only if the pigmentation is considered in conjunction with other tests.

The beta hemolytic activities and the coagulase positive reactions showed a closer correlation. Only a few strains of staphylococci showed beta hemolysis and were coagulase negative, while all alpha hemolytic strains were coagulase negative.

Serological agglutination cross reactions involving two groups of antisera yielded eight major antigenic groups. In many of these, both coagulase positive and negative strains were placed in one group.

The indirect fluorescent antibody test was inconclusive, while the direct test using fluorescein tagged immune serum showed some

correlation with the agglutination results, but with lesser specificity and higher degree of sensitivity. Many of the strains grouped by the agglutination tests were found to be in common groups on the basis of the fluorescent test.

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