Antigenic Study of Certain Slow Lactose Fermenting *Aerobacter cloacae* Cultures. (19812)

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Stuart, Wheeler, Rustigian and Zimmerman (1) reported an extensive biochemical and serologic study of paracolon bacilli in which they examined, among others, 140 strains having the general characteristics of Aerobacter except that they failed to ferment lactose or fermented this sugar only after several days. They called these strains paracolon Aerobacter and classified them into two divisions on the basis of their IMViC reactions. Each division was divided further into types on the basis of other biochemical reactions. In their first division, consisting of 48 cultures, they established 3 biochemical types and in their second division, containing 92 strains, 7 types were recognized. They designated these biochemical types simply by the numbers of typical strains in each type. The authors then prepared serums with a single representative strain from each of the biotypes except two, omitting one in each division, and performed agglutination tests in an effort to reveal the serologic homo- or heterogeneity of the biotypes. On the basis of these serologic studies they reported little serologic homogeneity in the biotypes of their first division, but implied that a high percentage of strains constituting each of the types in their second division were "antigenically identical or closely related." Since, in the preparation of their serums, Stuart and his co-workers used live cultures as antigens and tested the strains in each biotype only with the serum prepared with the type strain it is obvious that their study cannot be accepted as a basis for determining complete antigenic relationships. The methods employed by them are not given in sufficient detail to determine whether agglutination was of the O or the H type. It seems most probable that some titers were due chiefly to H, others to O antibodies. It appears from the findings of Stuart that considerable medical importance can be attached to certain biochemical types which have been classified in the second division. Type 32011 was incriminated as an inciting factor in gastroenteritis and type 37511 was on more than one occasion mistaken for a Salmonella. Both types were the most important members of the second division when considered on the basis of frequency of isolation from patients.

The cultural characteristics of the paracolon aerobacters are similar in many respects to normal *A. cloacae* cultures. In addition a number of investigations in the past have suggested that *A. cloacae*-like bacteria may be associated with a variety of human diseases (2-7). It was decided, therefore, that a more specific study of the antigens of the Stuart cultures would be desirable. Because of their similarity to normal *A. cloacae* cultures some strains of this species were introduced into the study for comparison purposes.

Methods. The following 32011 cultures were obtained from Dr. C. A. Stuart: SRC, 63511, 70811, 68411, C2, C3, C5, C6, and C7. Obtained from Stuart's collection also were cultures 32821, 35611, 37211, 37511, and 37711 which were described as differing biochemically from type 32011 but related antigenically. The following *A. cloacae* cultures were obtained from the American Type Culture Collection. ATC No. 222 (E. O. Jordan) and ATC No. 962 (M. Levine). Dr. W. H. Ewing supplied 2 paracolon aerobacter cultures, No. 2556 and 979 which had been received by the enteric laboratory, Communicable Disease Center, Atlanta, Ga., for identification as possible pathogens. All of the above cultures were observed for purity and evidence of serologic roughness. A number of other cultures obtained for use in this study could not be included because of an inability to obtain S forms. When tested biochemically, the Stuart cultures conformed to their original description. The Ewing cultures fermented lactose within 5 days and the *A. cloacae* cultures obtained from the American Type Culture Collection showed lactose fermentation in 48 hours. All of the cultures

fermented glycerol without gas formation within 5 days and all failed to ferment inositol within a 10-day observation period(8). In the serologic study of this group of cultures the well-known methods for the identification of O, H and K type antigens were used and attention was given to the possible existence in the cultures of the alpha type antigen of Stamp and Stone(9). *Thermostable somatic antigens* were prepared from 20-hour beef extract peptone broth cultures which had been heated at 100° C for 2½ hours. They were preserved by the addition of 0.4% formalin. *K type antigens* were prepared from living cultures grown for 12-17 hours on fresh beef extract agar slants containing 0.1% glucose. The slide agglutination technic was used for the demonstration of K type antigens. *H antigen* preparation was attended with some difficulty at the beginning of the study. It was discovered, however, that 3 or 4 rapid passages through a test tube column of semisolid agar 10 cm in depth (beef extract peptone broth with 0.2% agar) would produce motile cultures wholly satisfactory for agglutination tests and for immunization. Following motility enhancement cultures were inoculated into beef extract peptone broth and incubated for 16 hours. Cultures were killed and preserved by the addition of 0.4% formalin. In the preparation of *antiserums*, young rabbits weighing from 4 to 5 lb were given a series of 4 intravenous antigen injections in the amount of 0.5 cc, 1.0 cc, 1.5 cc, and 2.0 cc. Injections were given at 3- to 4-day intervals. One week after the last injection, the animals were bled. Serums were mixed with an equal volume of C. P. glycerol and stored. All serums were tested for the presence of alpha antibody by the employment of the Wakefield alpha strain. None of the serums used in this study contained alpha antibody.

Results. In our study of O antigens, 1:20 dilutions of the serum-glycerine mixture were made in 0.5% phenolized saline solution. These stock dilutions were the starting point for all titrations reported. Stock dilutions of seventeen O serums were prepared. Cross agglutination tests were conducted at a 1:40 dilution. Where cross agglutination was observed, titrations were started at 1:80 Table

TABLE I. Cross Agglutination of Heat Stable Antigens (2½ Hr at 100°C).

Antigens	Antiserums: ATC 962	ATC 222	SBO	68411	56211	Stuart's bio-type 32011 63511	70811	C3	C5	C6	C7	Stuart's Related bio-types 37511	35611	37711	32821	Ewing 2556	979
ATC 962	1280*	—															
ATC 222	—	1280	640														
SBO	—	640	640														
68411	—	—	—	1280													
56211	—	—	—	—	1280												
63511	—	—	—	—	—	640											
70811	—	—	—	—	—	—	2560										
C3	—	—	—	—	—	—	—	640									
C5	—	—	—	—	—	—	—	—	1280								
C6	—	—	—	—	—	—	—	—	—	1280	640						
C7	—	—	—	—	—	—	—	—	—	640	640						
37511	—	—	—	—	—	—	—	—	—	—	—	2560					
35611	—	—	—	—	—	—	—	—	—	—	—	—	1280				
37711	—	—	—	—	—	—	—	—	—	—	—	—	—	640	640	1280	640
32821	—	—	—	—	—	—	—	—	—	—	—	—	—	1280	1280	1280	1280
2556	—	—	—	—	—	—	—	—	—	—	—	—	—	1280	640	1280	1280
979	—	—	—	—	—	—	—	—	—	—	—	—	—	1280	640	1280	640

* Figure indicates reciprocal of highest dilution at which a visible agglutination was observed by unaided eye.
† Minus (—) indicates no agglutination observed at 1:40 or higher dilutions.

I shows the O antigen relationships within the paracolon aerobacter-*A. cloacae* group. It appears obvious from the table that the cultures included in Stuart's biotype 32011 are by no means homogeneous in respect to their O antigens. On the contrary, among the 9 strains 8 completely different O antigens were demonstrated. Strains C6 and C7, however, proved to be identical. One of the 32011 cultures, SRC, proved to have the same O antigen as the *A. cloacae* culture ATC 222. The four strains, 37711, 32821, 2556, and 979 proved also to be identical in their O antigens. Antigen relationships as stated were confirmed by reciprocal absorption tests. Culture ATC 962 proved to be unrelated to any of the paracolon Aerobacter cultures or to ATC 222.

H antigen relationships were investigated in a similar manner to that described for the thermostable somatic antigens. Seventeen H antiserums were prepared. Stock serum dilutions were made with 0.85% sodium chloride solution. Chloroform was added to the stock serum dilutions as a sterilizing and preserving agent(10). Cross titrations were performed beginning with a dilution of 1:40 and continuing to the titer of the serum. As will be observed in Table II the H antigens show a greater degree of interrelationship than was evident in the thermostable somatic antigens. One culture, 70811, gave no evidence of being related to any of the other cultures tested. The H antigens of cultures C6 and C7 appear to be identical and unrelated to the other cultures. ATC 222, ATC 962, and C3 also appear to be identical and unrelated to the other members of the group. The remaining eleven cultures show considerable H antigen relationship, but fall into three divisions as indicated by the enclosing brackets in Table II. The four strains of bracket 1 give cross reactions practically to the homologous titers of the serums not only with each other but also with the cultures of brackets 2 and 3. The 4 cultures of bracket 2 though giving good cross reactions with each other and with the bracket 1 strains, fail completely to cross with the 3 antigens of bracket 3. These latter give good reactions with the 4 members of bracket 1.

TABLE II. Cross Agglutination of H Antigens.

H antigens	H serums																
	1				2				3								
	SRC	37711	2556	979	68411	56211	63511	37511	35611	C5	32821	ATC 222	ATC 962	C3	C6	C7	70811
SRC	*5120*	2560	5120	2560	2560	2560	2560	5120	2560	2560	2560						
37711	5120	*5120*	5120	2560	2560	2560	2560	2560	2560	2560	2560						
2556	5120	5120	*5120*	5120	2560	2560	2560	2560	2560	2560	2560						
979	5120	5120	5120	*5120*	2560	2560	2560	2560	2560	2560	2560						
68411	2560	2560	2560	2560	*5120*	5120	5120	5120	—	—	—						
56211	2560	2560	2560	2560	5120	*5120*	5120	5120									
63511	5120	2560	2560	2560	5120	5120	*5120*	5120									
37511	2560	2560	2560	2560	5120	5120	5120	*5120*									
35611	2560	2560	2560	2560	5120	5120	5120	5120	*5120*	5120	5120						
C5	2560	2560	2560	2560	5120	5120	5120	5120	5120	*5120*	5120						
32821	2560	2560	2560	2560	5120	5120	5120	5120	5120	5120	*5120*						
ATC 222	—	—	—	—	—	—	—	—	—	—	—	*5120*	2560	2560			
ATC 962												2560	*5120*	5120			
C3												2560	5120	*5120*			
C6															*5120*	5120	
C7															5120	*5120*	
70811															—	—	*5120*

TABLE III. Phase Variation of Culture 2556.

H antigen		H serum 2556 Unabsorbed	Absorbed by 2556 (phase 1)	Absorbed by 2556 (phase 2)
2556		5120	2560	2560
	phase 1	2560	—	2560
	" 2	2560	2560	—

TABLE IV. Phase Variation of ATC 222.

H antigens		H serum ATC 222 Unabsorbed	Absorbed by ATC 222 (phase 1)	Absorbed by ATC 222 (phase 2)
ATC 222		5120	2560	2560
	phase 1	2560	—	2560
	" 2	2560	2560	—
	/962*	2560	—	2560
	/C3*	2560	—	2560
962		2560	—	2560
C3		2560	—	2560

* ATC 222/962, ATC 222/C3 indicates H phase of ATC 222 suppressed by serums 962 and C3.

These apparently anomalous results seem to be explainable only on the assumption that the strains of bracket 2 and those of bracket 3 are related each to a different H antigen of the cultures included in bracket 1. Such a relationship suggests the occurrence of phase variation in the flagellar antigens of bracket 1 cultures.

In an effort to reveal *phase variation* in the 4 cultures of bracket 1 H antigens were prepared from single colony isolations from culture 2556. These were used for absorbing 2556 antiserum. Tests with these absorbed serums indicated that the colonies were of two antigenic types in respect to their H antigens. Table III gives the results obtained with these absorbed serums and single colony antigens prepared from culture 2556. Subsequently culture 37711, SRC, and 979 and their homologous serums were similarily investigated with exactly the same results.

This related group of 11 cultures, therefore, appears to fall into 3 sub-groups. The first is composed of cultures SRC, 37711, 2556, and 979 (bracket 1) which are diphasic and identical to each other in both phases. The second sub-group (bracket 2) consists of cultures 68411, 56211, 63511, and 37511 which have H antigens identical to one of the phases occurring in bracket 1 cultures. Arbitrarily we shall call this antigen phase 1. The third sub-group, 35611, C5, and 32821 (bracket 3) contains an H antigen identical to that occurring in the other phase (phase 2) of the diphasic group.

Further investigations of the diphasic and monophasic cultures of this group were made employing the phase suppression technique. By this method it was found possible to obtain the desired phases from all of the diphasic cultures previously described. The results obtained were in every way similar to those given by the colony selection and serum absorption techniques mentioned above. These techniques were also applied to the remaining cultures of our series. Only one of these latter cultures, however, (culture ATC 222) was found to be diphasic. Cultures 962 and C3, appearing in Table II to be identical to ATC 222, were found to be monophasic and to possess only one of the H antigens of ATC 222. Table IV gives results demonstrating phase variation in culture ATC 222 and indicates the H antigenic relationships of that culture and cultures 962 and C3.

Cultures C6 and C7 were found to be identical, monophasic and unrelated to the other cultures. Culture 70811 proved to be monophasic also and unrelated to any of the other cultures studied.

TABLE V.
O and H Antigen Relationships Summarized.

Cultures	Thermostable antigens	H antigens
SRC	1	1:2*
ATC 222	1	3:4
ATC 962	2	3
68411	3	1
56211	4	1
63511	5	1
37511	6	1
C5	7	2
35611	8	2
32821	9	2
37711	9	1:2
2556	9	1:2
979	9	1:2
C3	10	3
C6	11	5
C7	11	5
70811	12	6

* This form is used to indicate the diphasic nature of the antigens. No implication is intended as to their group or specific nature.

The K antigens, characterized by their ability to inhibit O agglutination, include the thermolabile L and B envelope antigens and the capsular A type antigen. Our cultures were tested for these antigens by means of the slide agglutination technique using living cultures suspended in 0.85% sodium chloride solution. Varying dilutions of the homologous O serums were mixed with these bacterial suspensions and observed for evidence of agglutination. In all cases agglutination occurred normally. In fact in a few instances it appeared that living antigens were more sensitive to the O serums than the corresponding boiled antigens. These observations appeared to exclude the occurrence of the thermolabile L and B antigens in our cultures. To detect or rule out the A type antigens, however, requires a somewhat different technique, since antibodies against these thermostable antigens may be present in our O serums. Knipschildt(11) solved this problem by isolating from his A-containing coli cultures variant A minus forms. Later Vahlne(12) reported that some of his cultures isolated as A minus forms actually contained a small amount of A antigen, sufficient in fact, to cause inhibition of O agglutination. He further reported, however, that A antigen could be destroyed by heating at 120°C for 2 hours leaving the O antigens intact. We attempted both of these methods. We were unsuccessful in our attempts to isolate from our cultures colonies having the characteristics of Knipschildt's A minus forms. When autoclaved antigens were used to immunize animals we found that no demonstrable agglutinins appeared in the animals either for autoclaved or boiled antigens. The antigenic properties of our cultures appeared to be totally destroyed by this treatment. These results suggest the possibility that antigens in our boiled cultures (Table I) may be of the A type rather than true O antigens.

Discussion and conclusion. In a consideration of the experimental results reported here it appears obvious that the thermostable somatic antigens in this group of microorganisms are extremely heterogeneous. Seventeen cultures were examined and from this number, 12 somatic antigenic types were established. Nine cultures in this series represented the biotype 32011 and of this number eight contained distinct somatic antigens. Cultures C3, C5, C6, and C7 representing the 32011 type isolated from a gastroenteritis epidemic were found antigenically to belong in three distinct somatic groups. It seems improbable that the inciting cause of an epidemic would be represented by such a diversified group of antigenic types.

When one considers the flagellar antigens of this group it becomes apparent that Stuart's findings of near antigenic homogeneity in his 32011 cultures was in all probability due to the interrelationships of a few, but relatively commonly occurring, H antigens. The occurrence of phase variation in these antigens serves to increase the possibility of finding antigenic relationships in this group.

While a consideration of biochemical variants has been but a minor phase of this study, nevertheless it has been kept constantly in mind and especially in so far as it concerns lactose fermentation. No difficulty was encountered in isolating slow lactose fermenting variants from normal *A. cloacae* cultures when these were plated upon appropriate media. The obtaining of rapid lactose fermenters from slow fermenting cultures was attended with much more difficulty. Paracolon aerobacter cultures which have been isolated in the course of a search for enteric pathogens are normally selected because of their resemblance to non-lactose fermenting pathogens. Obviously colonies showing evidence of fermentation would not be isolated unless some special investigation was in progress. It would appear that it should now be possible to compare and determine by antigenic means whether lactose fermenting and non-lactose fermenting colonies obtained from a single individual are of the same antigenic type.

While no definite conclusions can be drawn from this study as to the relationships of paracolon aerobacter cultures of the biotype 32011 to normal *A. cloacae,* represented in this study by ATC 222 and 962, the experimental results indicate that these organisms have as much antigenic relationship to the normal forms as they have to each other. A definite conclusion in this matter awaits a more extensive

investigation of the antigens contained in a larger group of normal *A. cloacae* cultures.

The present investigation has shown that it is not only possible but wholly feasible to classify paracolon aerobacter cultures of the *A. cloacae* type by means of antigenic analysis. It would appear premature to establish an antigenic schema for this group though data summarized in Table V suggest such a system could be devised.

In the future when organisms of the *A. cloacae* type are encountered, especially if associated with disease, it would seem desirable that such bacteria should be antigenically typed. Such a procedure should increase the probability of determining the pathogenic properties of this group.

Summary. 1. Paracolon aerobacters represented by biotype 32011 and related cultures were subjected to antigenic study and comparison. 2. A single biotype was found to be extremely heterogeneous in respect to its thermostable somatic antigens. 3. The occurrence of phase variation in the flagellar antigens of this group is reported and H antigenic relationships are determined. 4. It is pointed out that the antigenic system of classification can be profitably utilized in further investigation of this group.

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Heidenhain Pouch Secretory Response as Affected by Gastrojejunostomy to the Main Stomach.* (19813)

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Gastrojejunostomy has waned in popularity as a surgical procedure for the cure of peptic ulcer during the past 2 decades. Gastrojejunostomy was formerly advocated as a means of neutralizing the gastric secretion. We have not found any reports in the literature dealing with a quantitative study of the acid secretory response of the gastric mucosa to this procedure. We therefore undertook the following experiment.

Method. A Heidenhain pouch was produced and drained externally by a water tight nylon cannula in each of 5 healthy mongrel dogs. Following recovery from the anesthesia (intravenous Nembutal), the dogs were placed in individual cages in a special room. Fresh water was provided at all times. Milk and soft food were given for the first 3 or 4 postoperative days and, thereafter, the dogs were given a single daily feeding of 100 g cooked

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