THE SEROLOGIC AGGLUTINATION OF THE OBLIGATE ANAEROBES CLOSTRIDIUM PARAPUTRIFICUM (BIENSTOCK) AND CLOSTRIDIUM CAPITOVALIS (SNYDER AND HALL)

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Early workers, LeClainche and Vallee (1900), Bachmann (1904), Rocchi (1911), Markoff (1911), Meyer (1915) and others, differentiated *Clostridium oedematismaligni* (Koch), Ghon and Sachs bacillus (*Vibrion septique*), and the "Rauschbrandbacillus" (*Clostridium chauvei*) by agglutination. On the other hand, Zeissler (1919) and Heller (1920) condemned the agglutination test for sporulating anaerobes because of the occurrence of spontaneous agglutination and cross-reactions of certain sera with unrelated species.

More recently, however, several investigators have used serologic agglutination tests in the identification of anaerobic bacilli without encountering either spontaneous agglutination or non-specific cross-agglutination, as witness the work of Goss, Barbarin and Haines (1921) with *Cl. chauvei* and the *Vibrion Septique*, Starin and Dack (1923) with *Clostridium sporogenes*, *Clostridium botulinum*, and *Clostridium putrificum*, Hall and Stark (1923) with *Cl. sporogenes*, Hall and Scott (1931) with *Clostridium Sordellii*, Duffett (1935) with *Clostridium carnis*, and McCoy and McClung (1935) with *Clostridium acetobutylicum*.

Agglutination methods, moreover, have demonstrated serological groups in several anaerobic species, particularly in *Clostridium tetani* by Tulloch (1919), Bauer (1924), Fildes (1925), and Coleman and Gunnison (1928) and in *Cl. botulinum* by Schoenholz and Meyer (1923).

In the light of these facts it was thought of interest to report the agglutination reactions which not only differentiated sharply *Clostridium paraputrificum* from *Clostridium capitovalis*, but also established serological subgroups in *Cl. paraputrificum*. Acid agglutination was encountered and especially studied because of its rôle in giving false reactions in these anaerobic agglutination tests.

The cultural characteristics separating these two non-pathogenic, terminal, oval-spored anaerobes isolated from intestinal contents and postmortem material were reported by Hall and Snyder (1934) and Snyder and Hall (1935). They showed that *Cl. paraputrificum* was obligately saccharolytic and probably identical with Escherich's "Kopfchenbakterien" (1886) and Rodella's Bacillus III (1902), but had been adequately described and named by Bienstock (1906). A careful search of the literature, however, failed to show any organism identical with the one named *Cl. capitovalis*, which differed from *Cl. paraputrificum* in being mildly proteolytic.

METHODS

Agglutinating sera of a high titer were prepared in rabbits by a series of nine or more subcutaneous injections of 2.0 cc. of forty-eight-hour glucose broth cultures at two to five-day intervals. Prior to the first inoculations no normal agglutinins were demonstrated in the rabbits. When a series of inoculations was finished, ten days or more were allowed to lapse before the rabbit was bled from the marginal ear vein for a preliminary titration of the serum. If this titration was found to be satisfactory, larger quantities of serum were obtained by cardiac bleeding.

Forty-eight-hour glucose broth cultures in Hall's constricted tubes were used for the agglutination tests. The cultures were filtered through cotton and diluted to an opacity of 300 on the turbidity scale recommended in "Standard Methods of Water Analysis" (1923). Titrations of the various sera were carried out by diluting the sera 1:10 and doubling the dilutions up to 1:2560. The addition of an equivalent amount of bacterial culture then

gave serum dilutions from 1:20 to 1:5120. The mixtures were incubated at 37°C. Because of the apparently slow agglutination the first tests were recorded at 12 hours, but it was found later that seven hours were sufficient for complete flocculation.

DATA

For the preliminary analysis of eighteen strains of *Cl. paraputrificum* and seven strains of *Cl. capitovalis*, a fecal strain of *Cl. paraputrificum*, no. 4453, was selected. After a satisfactory agglutinating serum was prepared, tests were carried out against the other strains according to the technique outlined. The results are given in table 1.

Table 1 shows that all strains of *Cl. paraputrificum* were partially or completely agglutinated in a serum dilution of 1:40. Of these strains, however, nos. 1538, 1545, 3698, and 4388 were flocculated as strongly as the homologous culture, while strains 3572 and 4065were even more strongly flocculated, indicating an antigenic subdivision in the species *Cl. paraputrificum*. The *Cl. capitovalis* strains, except no. 3904 which was not agglutinated, showed traces of agglutination in all tubes including the saline controls and complete flocculation in a 1:320 dilution with strain. 674. These positive reactions could not be explained at this time.

The presence of serological groups in *Cl. paraputrificum* was further shown by preparing an agglutinating serum against a strain only slightly agglutinated by antiserum 4453; strain 4042 was selected. This serum was tested against the eighteen *Cl. paraputrificum* and seven *Cl. capitovalis* strains. It was found that strains 3978, 3941, 3909, and 4351 which were not markedly agglutinated by antiserum 4453 were flocculated as strongly as the control, or even more strongly, while strains 1538, 1545, 3698, 3572, 4388, and 4065 brought down strongly by antiserum 4453 were weakly agglutinated by antiserum 4042. This confirmed the presence of at least one antigenic subdivision in the species *Cl. paraputrificum*. Cross-agglutination with *Cl. capitovalis* strains showed partial flocculation in all tubes including the saline con-

TABLE 1

Cross-agglutination tests of Cl. paraputrificum and Cl. capitovalis strains with Cl. paraputrificum antiserum 4453

	STRAIN NUMBER	SERUM DILUTIONS									CONTROL
		1:20	1:40	1:80	1:160	1:320	1:640	1:1280	1:2560	1:5120	
Cl. paraputrificum	3978	+++	+++	++	−	−	−	−	−	−	−
	491	++++	++++	++++	+++	++	−	−	−	−	−
	1538	++++	++++	++++	+++	+++	++	+	−	−	−
	1545	++++	++++	++++	++++	++++	+++	++	+	−	−
	3572	++++	++++	++++	++++	++++	++++	++++	++++	+++	−
	3638	++++	++++	+++	++	−	−	−	−	−	−
	3698	++++	++++	+++	+++	++	−	−	−	−	−
	3941	+++	++	+	−	−	−	−	−	−	−
	3953	++++	++	−	−	−	−	−	−	−	−
	4042	+++	++	−	−	−	−	−	−	−	−
	3909	++++	++	+	−	−	−	−	−	−	−
	3945	+++	+++	++	+	−	−	−	−	−	−
	4013	++++	+++	++	+	−	−	−	−	−	−
	4104	++++	++++	+++	+	−	−	−	−	−	−
	4351	++	++	++	−	−	−	−	−	−	−
	4388	+++	+++	+++	++	+	+	+	+	−	−
	4453*	++++	++++	++++	++++	++++	+++	+++	+	+	−
	4065	++++	++++	+++	+++	++	++	++	+	+	−
Cl. capitovalis	137	+	+	+	+	+	+	+	+	+	+
	2076	+	+	+	+	+	+	+	+	+	+
	3543	+	+	+	+	+	+	+	+	+	+
	3904	−	−	−	−	−	−	−	−	−	−
	4236	+	+	+	+	+	+	+	+	+	+
	613	+	+	+	+	+	+	+	+	+	+
	674	+	+	+	+	++++	+	+	+	+	+

* Homologous strain.

trols of strains 4236 and 613, but not with the remaining five strains. These positive results could not be explained at the time of reading.

While these tests were being made, an agglutinating serum was prepared against a strain of *Cl. capitovalis*, no. 137. Cross-agglutination tests were carried out with this and the remaining strains of *Cl. paraputrificum* and *Cl. capitovalis*. All strains of *Cl. capitovalis* agglutinated to nearly the same degree as the control. The reactions of the *Cl. paraputrificum* strains, however, were of considerable interest because all strains showed some agglutination in a 1:20 serum dilution, many in a 1:40 dilution, and a few at 1:80. The unusual thing appeared to be the sharp end-point rather than the tapering off, ordinarily encountered in specific agglutination. These agglutinations were suspected to be the results of the action of the acid produced in the glucose broth by the vigorous fermentation of this sugar by *Cl. paraputrificum*, and corresponded to the type of acid agglutination described by Michaelis (1911) and others. Colorimetric tests with brom-cresol-green showed all *Cl. paraputrificum* strains produced a terminal acidity in glucose broth of pH 4.4 to 4.8, while *Cl. capitovalis* strains were slightly higher at pH 4.9 to 5.1.

The accuracy of this hypothesis was tested by adding N/10 NaOH to the glucose broth cultures until neutrality was approximated as tested by brom-thymol-blue on aliquot samples. These neutralized cultures were then tested against antiserum 137 in parallel with a series of acid cultures as controls. The acid series gave essentially the same results as before. The results with the neutralized cultures are listed in table 2.

Table 2 shows that while neutralization lowered the titer limits of the homologous and related *Cl. capitovalis* strains, it almost eliminated the pseudo-reactions of the *Cl. paraputrificum* strains as previously encountered. Despite the apparent specificity of these results, a question arose over the possible influence of the salts formed in the neutralization process.

To avoid this factor the cultures were grown in neutral peptone infusion broth in Hall's constricted tubes. While growth was not as heavy as in glucose broth, it was usually sufficient to carry out

TABLE 2

Cross-agglutination tests of Cl. paraputrificum and Cl. capitovalis strains with Cl. capitovalis antiserum 157

Forty-eight-hour glucose broth cultures neutralized with N/10 NaOH

	STRAIN NUMBER	SERUM DILUTIONS									CONTROL
		1:20	1:40	1:80	1:160	1:320	1:640	1:1280	1:2560	1:5120	
Cl. paraputrificum	3978	+	+	−	−	−	−	−	−	−	−
	491	−	−	−	−	−	−	−	−	−	−
	1538	−	−	−	−	−	−	−	−	−	−
	1535	−	−	−	−	−	−	−	−	−	−
	3572	−	−	−	−	−	−	−	−	−	−
	3638	−	−	−	−	−	−	−	−	−	−
	3698	+	+	−	−	−	−	−	−	−	−
	3941	−	−	−	−	−	−	−	−	−	−
	3953	−	−	−	−	−	−	−	−	−	−
	4042	−	−	−	−	−	−	−	−	−	−
	3909	−	−	−	−	−	−	−	−	−	−
	3945	−	−	−	−	−	−	−	−	−	−
	4013	−	−	−	−	−	−	−	−	−	−
	4104	−	−	−	−	−	−	−	−	−	−
	4351	−	−	−	−	−	−	−	−	−	−
	4388	−	−	−	−	−	−	−	−	−	−
	4453	−	−	−	−	−	−	−	−	−	−
	4065	−	−	−	−	−	−	−	−	−	−
Cl. capitovalis	137*	++++	++++	++++	++++	++++	++	+	−	−	−
	2076	++++	++++	++++	++++	+++	++	+	−	−	−
	3543	++++	++++	++++	+++	++	++	−	−	−	−
	3904	++++	++++	++++	++++	++	+	−	−	−	−
	4236	++++	++++	++++	++++	++++	++	++	−	−	−
	613	++++	++++	++++	++++	+++	++	+	−	−	−
	674	++++	++++	++++	+++	+++	++	+	−	−	−

* Homologous strain.

TABLE 3

Cross-agglutination tests with Cl. paraputrificum and Cl. capitovalis strains with Cl. paraputrificum antiserum 4453

Forty-eight-hour peptone infusion broth cultures used

	STRAIN NUMBER	SERUM DILUTIONS									CONTROL
		1:20	1:40	1:80	1:160	1:320	1:640	1:1280	1:2560	1:5120	
Cl. paraputrificum	3978	+++	++	+	−	−	−	−	−	−	−
	491	++++	+++	++	−	−	−	−	−	−	−
	1538	++++	++++	++	−	−	−	−	−	−	−
	1545	+++	++	−	−	−	−	−	−	−	−
	3572	++++	++++	++++	++++	++++	++++	++++	++	−	−
	3638	+++	++	−	−	−	−	−	−	−	−
	3698	++++	++++	++++	++++	++++	++++	−++++	+++	++	−
	3941	++	−	−	−	−	−	−−	−	−	−
	3953	+++	++	−	−	−	−	−−	−	−	−
	4042	++++	++	−	−	−	−	−	−	−	−
	3909	++++	++	−	−	−	−	−	−	−	−
	3945	++++	++++	−	−	−	−	−	−	−	−
	4013	+++	++	−	−	−	−	−	−	−	−
	4104	+++	++	−	−	−	−	−	−	−	−
	4351	++++	++	−	−	−	−	−	−	−	−
	4388	++++	++++	++	−	−	−	−	−	−	−
	4453*	++++	++++	++++	++++	+++	++	−	−	−	−
	4065	+++	++	++	−	−	−	−	−	−	−
Cl. capitovalis	137	−	−	−	−	−	−	−	−	−	−
	2076	−	−	−	−	−	−	−	−	−	−
	3543	−	−	−	−	−	−	−	−	−	−
	3904	−	−	−	−	−	−	−	−	−	−
	4236	−	−	−	−	−	−	−	−	−	−
	613	−	−	−	−	−	−	−	−	−	−
	674	−	−	−	−	−	−	−	−	−	−

* Homologous strain.

the agglutination tests. At the end of forty-eight hours the hydrogen-ion concentration of the peptone infusion broth cultures was not appreciably changed. Cross-agglutination tests were then carried out with antiserum 137. A control series of acid cultures was also carried out with results similar to those previously described. Even sharper differentiation between the strains of *Cl. capitovalis* and *Cl. paraputrificum* was obtained with the use of peptone infusion broth cultures. The apparent antigenic homogeneity of *Cl. capitovalis* was retained.

The process was repeated with *Cl. paraputrificum* agglutination serum 4453. Control acid cultures gave results similar to those in table 1 with *Cl. paraputrificum* strains, and partial agglutination in the 1:20 and 1:40 serum dilutions with *Cl. capitovalis* strains 137, 2076, 4236, 613, and 674. The results with the peptone infusion broth cultures are listed in table 3.

The data in table 3 confirm the sharp demarcation serologically between the two species when the disturbing acid factor was eliminated. The results of these agglutination tests with *Cl. paraputrificum* demonstrate again the existence of antigenic subdivisions in the species as shown in table 1.

DISCUSSION

The evidence submitted showed that the agglutination test separated *Cl. paraputrificum* and *Cl. capitovalis* as distinct species which confirmed the previously reported cultural studies on these two morphologically similar anaerobes. The larger number of *Cl. paraputrificum* strains afforded a better opportunity to establish antigenic subdivisions in the species than the smaller number of *Cl. capitovalis* strains, but no attempt has yet been made to confirm by receptor analysis the antigenic heterogeneity in *Cl. paraputrificum* as established by the agglutination tests. Cross-agglutination tests with other non-pathogenic, terminal, oval-spored anaerobes, *Clostridium cochlearium*, *Clostridium tetanomorphum*, *Clostridium caloritolerans*, and *Clostridium putrificum*, were all negative.

This study served to determine roughly the terminal acidity above which broth cultures should not be used in agglutination

tests. Colorimetrically the glucose broth cultures of *Cl. paraputrificum* strains were under pH 4.8 while *Cl. capitovalis* strains were usually slightly above. Thus the critical point for the non-specific acid agglutination of broth cultures of these two species was apparently close to pH 4.8. Subsequent tests with broth suspensions of *Cl. paraputrificum*, *Cl. capitovalis*, and other anaerobic species showed that the broth rather than the organisms determined this type of acid agglutination.

It is obvious that the use of glucose broth cultures in agglutination tests with sporulating anaerobes may lead to spurious results, particularly if the hydrogen-ion concentration is overlooked and increases to give pH values below 5.0. This difficulty may be overcome in two ways; (1) by using neutralized glucose broth cultures, and (2) by growth of cultures in media containing so little carbohydrate that the pH is not significantly altered.

SUMMARY

Clostridium paraputrificum and Clostridium capitovalis were shown serologically to be distinct species.

Non-specific acid agglutinations were shown to occur when glucose broth cultures of these anaerobes with terminal acidity below pH 5.0 were used in agglutination tests.

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