BACTERIOPHAGE STUDIES ON THE HEMORRHAGIC SEPTICEMIA PASTEURELLAE

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The group of hemorrhagic septicemia pasteurellae as presently classified includes two species, Pasteurella multocida and Pasteurella haemolytica (Breed et al., 1948). P. multocida has been subdivided into three major groups on the basis of xylose, arabinose, and dulcitol fermentations (Rosenbusch and Merchant, 1939), but at least four serological groups exist within this species (Carter and Byrne, 1953). P. haemolytica strains have been placed into two groups according to lactose fermentation, while in contrast, three serological types have been demonstrated (Florent and Godbille, 1950). In addition, numerous organisms have been reported that obviously belong in this group but differ slightly from the classical descriptions. These have been assigned tentatively new specific names such as Pasteurella mastiditidis (Marsh, 1932) and Pasteurella pneumotropica (Jawetz, 1950).

P. multocida bacteriophage has been reported by Broulin (1926, 1927, 1929), D’Herelle (1921, 1926), and by Lazarus and Gunnison (1947), while Brigham and Rettger (1953), who suggested the use of these viruses in taxonomic studies on the genus Pasteurella, were unable to effect their isolation.

It was believed that bacteriophage studies on the hemorrhagic septicemia pasteurellae would serve two purposes: first, to provide additional information bearing upon the taxonomic interrelationships within this group, and second, to assess the practicability of devising a phage typing scheme for this widespread group of animal pathogens.

MATERIALS AND METHODS

One hundred forty-one cultures of hemorrhagic septicemia pasteurellae obtained in the United States and from Canada, England, and Belgium were studied.

The basal culture medium, which consisted of Albimi peptone M (2.0 per cent), Albimi yeast autolysate (0.2 per cent), NaCl (0.5 per cent), hemin (0.5 mg per cent), thiamin hydrochloride (0.1 mg per cent), nicotinamide (0.1 mg per cent), and calcium pantothenate (0.1 mg per cent), was adjusted to pH 7.4 with 10 N NaOH and autoclaved as broth or with 1.5 per cent Albimi agar. Fermentation reactions were determined after 5 days’ incubation in cystine tryptase agar (BBL) containing 1.0 per cent carbohydrate and supplemented with 0.1 mg per cent each of thiamin hydrochloride, nicotinamide, and calcium pantothenate (Jordan, 1932; Vera, 1950). Indole formation was tested after 48 hours’ growth in basal broth using Ehrlich’s reagent. Hemolysis was tested on 5 per cent sheep blood basal agar plates.

Bacteriophages were isolated from these organisms by the methods of Fisk (1942) and Warner (1950).

RESULTS

The biochemical reactions of the strains tested are summarized in table 1. In addition to the more prevalent lactose negative strains of P. multocida, two lactose positive organisms were found which correspond biochemically to P. pneumotropica. Among the P. haemolytica strains, nonhemolytic representatives were observed which show the biochemical pattern of typical P. mastiditidis.

Attempts to isolate P. multocida phages by the Fisk method, as reported previously by Lazarus and Gunnison (1947), proved successful. However, parallel isolation experiments following the Warner method failed to reveal the presence of a number of known phage systems. The Fisk method involves spotting of whole culture filtrates directly onto agar layer plates of the test strains, while in the Warner procedure a mixture of culture filtrates is passed serially in broth on the bacterial cultures before test spotting on the agar plates.
Influence of stock bacteriophage strains. After tubes were claved, of either solution mastiditis Pasteurella was supplied by being sensitive but which was lacking in broth but which was being supplied by the agar. Accordingly, tubes of basal broth were supplemented, after autoclaving, with 5 per cent of a sterile 2 × 10⁻² M solution of either CaCl₂, MgSO₄, or MnCl₂ and inoculated with known sensitive Pasteurella multocida strains. After two hours' incubation, 0.1 ml of stock bacteriophage was added to each tube, and these tubes were incubated overnight with parallel control tubes not containing phage. The presence of lysis was determined visually by comparing the turbidity of the experimental and control tubes (table 2).

Apparently certain P. multocida bacteriophages require calcium ions as a lysis cofactor, while for others, magnesium ions also will fill this role. In a few instances, no added cofactor is required, while in two cases manganous ions appear to inhibit bacterial lysis.

The activity of sixteen P. multocida bacteriophages and one P. haemolytica phage against the pasteurella strains was tested by a modification of the method of Craigie and Yen (1938). Three ml of melted 0.8 per cent basal agar were seeded

### Table 1

<table>
<thead>
<tr>
<th>Species Name</th>
<th>Number of Strains Tested</th>
<th>Biochemical Reactions</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Growth on Mac-</td>
</tr>
<tr>
<td>Pasturella multocida</td>
<td>113</td>
<td>−</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>−</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>−</td>
</tr>
<tr>
<td>Pasturella pneumotropica</td>
<td>1</td>
<td>−</td>
</tr>
<tr>
<td>Pasturella haemolytica</td>
<td>6</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>+</td>
</tr>
<tr>
<td>Pasturella mastiditis</td>
<td>5</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>+</td>
</tr>
</tbody>
</table>

A = Acid reaction; 0 = no acid reaction.

* Difco.

Preliminary investigations of this phenomenon revealed that bacteriophage could be recovered without change in titer from the supernate of a known sensitive culture in basal broth after eight hours' incubation. It appeared, therefore, that the demonstration of bacteriophage depended upon the presence of an absorption cofactor that was lacking in broth but which was being supplied by the agar. Accordingly, tubes of basal broth were supplemented, after autoclaving, with 5 per cent of a sterile 2 × 10⁻² M solution of either CaCl₂, MgSO₄, or MnCl₂ and inoculated with known sensitive P. multocida strains. After two hours' incubation, 0.1 ml of stock bacteriophage was added to each tube, and these tubes were incubated overnight with parallel control tubes not containing phage. The presence of lysis was determined visually by comparing the turbidity of the experimental and control tubes (table 2).

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### Table 2

<table>
<thead>
<tr>
<th>Number of Phages Tested</th>
<th>Basal Broth with 10⁻² M:</th>
<th>Indole</th>
<th>Hemolysis</th>
<th>Glucose</th>
<th>Sucrose</th>
<th>Lactose</th>
<th>Maltose</th>
</tr>
</thead>
<tbody>
<tr>
<td>3</td>
<td>−</td>
<td>−</td>
<td>A</td>
<td>A</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>−</td>
<td>+</td>
<td>A</td>
<td>A</td>
<td>A</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>+</td>
<td>+</td>
<td>A</td>
<td>A</td>
<td>0</td>
<td>A</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>+</td>
<td>+</td>
<td>A</td>
<td>A</td>
<td>0</td>
<td>A</td>
<td></td>
</tr>
</tbody>
</table>

+ = Lysis; − = no lysis.

### Table 3

<table>
<thead>
<tr>
<th>Bacteria Tested for Sensitivity*</th>
<th>Phages Isolated From:</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pasteurella multocida...</td>
<td>84/118</td>
</tr>
<tr>
<td>Pasteurella pneumotropica...</td>
<td>0/1</td>
</tr>
<tr>
<td>Pasteurella haemolytica...</td>
<td>0/16</td>
</tr>
<tr>
<td>Pasteurella mastiditis...</td>
<td>0/6</td>
</tr>
</tbody>
</table>

* Figures = no. of strains sensitive/no. tested.
with 0.3 ml of a broth culture of the test strain, and this mixture was poured over the surface of a basal agar plate. After hardening, dilutions of the various phages were spotted onto the surface of the inoculated plate. After incubating overnight at 35 C the presence of phage activity was observed (table 3).

**DISCUSSION**

The hemorrhagic septicemia pasteurellae are small, gram negative, catalase positive rods that reduce nitrate to nitrite and form acid in glucose, sucrose, and other carbohydrates (Breed et al., 1948). These organisms show variation as to growth on MacConkey agar, formation of indole, hemolysis, and fermentation of lactose, maltose, and some other carbohydrates. Typical strains of *P. multocida* do not grow on MacConkey agar, are indole positive, and are lactose and maltose negative, while stains of *P. haemolytica* grow on MacConkey agar, are indole negative, and lactose and maltose positive. Classification of strains which differ in one or more ways from these classical patterns presents a problem that has not to date been definitively resolved by serological means. Hemolysis, the primary criterion for establishing the species *P. haemolytica*, is apparently a variable phenomenon (Florent and Godbille, 1950), while lactose fermentation is a rather weak delayed type of reaction. Further, a few strains of the lactose positive group (*P. pneumotropica* and *P. mastitidis*) failed to show this capacity, and several lactose positive strains of *P. multocida* were observed (see table 1). Finally, in considering the taxonomic interrelationships of the hemorrhagic septicemia pasteurellae, it is well to keep in mind the statement of Meyer (1948) that “The innumerable strains composing the group fluctuate easily and continuously in physiologic functions, antigenic structure, fermentation capacity and pathogenic ability.”

It would seem, then, that these organisms could be divided more conveniently into two species on the basis of only indole formation and growth on MacConkey agar. *P. multocida* would include those strains that do not grow on MacConkey agar, are indole positive, are nonhemolytic, and are usually lactose negative. *P. haemolytica* would comprise strains which grow on MacConkey agar, are indole negative, are sometimes hemolytic, and are usually lactose positive. Following this suggestion, differences in the fermentation of a single carbohydrate (e.g., lactose or maltose) or the presence of hemolysis does not necessitate the establishment of a new species within this very closely related group of microorganisms. Although *P. haemolytica* would now include a number of nonhemolytic strains, the species name is retained provisionally to avoid the confusion that might result from the introduction of still another name into the group of animal pasteurellae.

A number of *P. multocida* bacteriophages were shown to require calcium or magnesium ions as an absorption cofactor. The failure of Brigham and Rettger (1935) to demonstrate the phages may have been due to a deficiency of these ions in their broth medium, while Lazarus and Gunnesson (1947), using only the agar plate method of phage isolation, apparently were not concerned with this phenomenon.

Results of the studies on the host range of pasteurella bacteriophage do not mitigate against the broadening of the species definitions suggested above since one of the lactose positive *P. multocida* strains was sensitive to a bacteriophage active against several of the more common lactose negative organisms of this species.

Experiments reported here indicating that 84 of 118 (71 per cent) of the *P. multocida* strains were sensitive to one or more of 16 bacteriophages certainly show that a phage typing scheme would be practicable. This epidemiological tool could be of value in advancing our knowledge of the carrier state, distribution, and transmission of this widespread group of animal pathogens.

**SUMMARY**

It is suggested on the basis of cultural characteristics, biochemical reactions, and bacteriophage sensitivity that the hemorrhagic septicemia pasteurellae might be divided into two rather broad species categories, *Pasteurella multocida* and *Pasteurella haemolytica*, on the basis of indole formation and growth on MacConkey agar.

A number of *P. multocida* bacteriophages studied were shown to require calcium or magnesium ions as a cofactor for lysis in broth.

Eighty-four of 118 (71 per cent) *P. multocida* strains were found to be sensitive to one or more of 16 bacteriophages.

**REFERENCES**

Breed, R. S., Murray, E. G. D., and Hutcheson, A. P. 1948 *Bergey's manual of determina-


