Serological Studies on *Actinobacillus actinomycetem-comitans*

G. PULVERER and H. L. KO

Institute of Hygiene, University of Cologne, 5 Cologne, Germany

Received for publication 28 September 1971

One hundred strains of *Actinobacillus actinomycetem-comitans* were examined serologically by determining their agglutination reactions with six absorbed antisera. Twenty-four reaction patterns were found, and the results were reproducible. The agglutinating antigens were heat-stable. Precipitation reactions were also studied by means of the Ouchterlony agar-gel diffusion technique with Fuller and acetone extracts. The agglutination test was considered preferable for epidemiological and ecological investigations on *A. actinomycetem-comitans*.

We recently reported our studies on the fermentative capabilities of *Actinobacillus actinomycetem-comitans* (3). The present report deals with serological studies on 100 strains of *A. actinomycetem-comitans*.

**MATERIALS AND METHODS**

**Strains.** The 100 strains studied were selected from the strains described previously (3). Strain 3349, described in a prior publication (1), was included for comparative purposes.

**Preparation of antigen suspension.** Strains of *A. actinomycetem-comitans* were cultivated for 4 days on the surface of the following medium: Casitone, 12 g; Difco yeast extract, 12 g; KH₂PO₄, 4 g; MgSO₄, 7H₂O, 1 g; agar, 25 g; and distilled water, 1,000 ml; the pH was 7.2. Colonies were suspended in phenolic (0.5%) saline. This suspension was treated for 5 min in an ultrasonic disintegrator (MSE, 500 w) and standardized to the density of a McFarland nephelometer tube no. 5. This antigen suspension was used for immunization, for agglutination tests, and for preparation of precipitating antigens.

**Antiserum preparation.** Rabbits were immunized with the *A. actinomycetem-comitans* suspension described above. The immunization schedule was as follows: 0.5, 1.0, 1.5, 1.5, 2.0, 2.0, and 2.0 ml intravenously at 2-day intervals, followed by a 1-week interruption and then three further intravenous injections of 2.0, 2.5, and 2.5 ml at 2-day intervals. Blood was obtained by cardiac puncture. Serum was stored at −40 C.

**Agglutination test.** Rabbit antiserum was first diluted 1:20 in phosphate buffer (KH₂PO₄, 1.45 g; Na₂HPO₄, 7.6 g; NaCl, 4.8 g; distilled water, 1,000 ml). Two drops of the antigen suspension were added to 0.5 ml of each serum dilution. Tubes were shaken thoroughly, and were incubated for 24 hr at 37 C (first reading) and for another 24 hr at 22 C (second reading). Serum dilutions were considered positive when a clear agglutination reaction could be seen at the second reading. Positive and negative controls were always carried out.

**Serum absorption.** Rabbit serum, diluted 1:5 in phosphate buffer, was mixed with an equal volume of the antigen suspension. After agitation for 1 hr at 37 C and for an additional 4 hr at 37 C, the mixture was centrifuged at 4,000 × g for 20 min. The clear supernatant fluid was used for agglutination tests.

**Precipitation test.** The Ouchterlony agar-gel diffusion test was used with two different antigen preparations: Fuller extract and acetone extract.

**Fuller extract.** The antigen suspension was washed twice in physiological saline and then centrifuged (20 min at 4,000 × g). A 1-g amount of the sediment was added to 5 ml of formamide and heated in an oil bath for 20 min at 160 C. After cooling, 2.5 ml of an alcohol-HCl solution (5 ml of 1 N HCl plus 95 ml of 96% ethanol) was added, and 2 hr later the mixture was centrifuged (20 min at 4,000 × g). The supernatant fluid was mixed with an equal volume of acetone and stored overnight in a refrigerator at 4 C. After centrifugation, the supernatant fluid was discarded and the sediment was suspended in 1 ml of saline. The pH was adjusted to 7.2.

**Acetone extract.** A 50-ml amount of normal broth (meat extract, Oxoid, 10 g; peptone Brunengräber S, 12 g; NaCl, 3 g; K₂HPO₄, 2 g; distilled water, 1,000 ml; pH 7.5) was heavily (material from five plates) inoculated with *A. actinomycetem-comitans* and incubated for 3 weeks at 37 C. After centrifugation for 30 min at 1,000 × g, two volumes of cold acetone were added to one volume of the clear supernatant fluid, and this mixture was refrigerated at 4 C overnight. Centrifugation for 20 min at 4,500 × g followed, and the sediment was dissolved in 1 ml of distilled water.

**Agar-gel diffusion technique.** Three slides were
placed in a case (LKB Sweden 6801 A) and covered with 10 ml of agar [1 g of Reinagar Behringwerke was dissolved in 100 ml of a mixed solution of 75 ml of distilled water and 25 ml of Veronal buffer (LKB Veronal; ionic strength, 0.1; pH 8.6); melted phenol (0.25 ml) was added as a preservative]. The gel was allowed to harden; then five holes were cut out with an LKB Sweden gel punch type 6808 A. The central hole was filled with antisera; the peripheral holes were filled with the antigen preparations. After 24 hr at 22 °C, precipitation bands were observed. Photos were taken after staining with amido black (E. Merck, Darmstadt, Germany).

RESULTS

Agglutination test. Six antisera were prepared by immunizing rabbits with A. actinomy cetem-comitans strains 3349, 363, 4153, 7400, 64, and 87. Since these antisera were not monospecific, absorption was required. As shown in Table 1, the absorbed antisera reacted only with their homologous strains. The agglutination titers ranged from 1:160 to 1:320.

One hundred strains of A. actinomy cetem-comitans were examined by means of a tube agglutination test, with the use of six absorbed antisera (Table 2). A reaction was considered positive when agglutination was seen at least in an antisera dilution of 1:20. Of the 100 strains, 58 reacted only with a single antisera; 25 strains reacted positively with two, 8 strains with three, 5 strains with four, 1 strain with five, and 2 strains with all six antisera. Only one strain could not be agglutinated with any of the six antisera. Altogether, 24 different reaction patterns were observed.

Heat resistance of the A. actinomy cetem-comitans antigens was determined by use of the six homologous strains. The antigen suspensions were heated at 60 °C for 30 min, at 100 °C for 30 and 60 min, and at 120 °C for 30 min. The results obtained demonstrated that the agglutination antigens of A. actinomy cetem-comitans are heat-stable. All six preparations heated at 100 °C for 60 min yielded a positive agglutination test. The agglutination reactions actually appeared stronger after heat treatment. Preparations treated at 120 °C could not be evaluated because of spontaneous agglutination. When absorbed antisera were used, antigens treated for 60 min at 100 °C yielded the same agglutination spectrum as nonheated preparations.

When different media were used for cultivation of A. actinomy cetem-comitans strains (above medium; DST-medium, Oxoid; agar medium with 5% sheep blood), no changes were seen in the antigen spectrum. These studies were done with six randomly selected A. actinomy cetem-comitans strains.

Precipitation test. The results of the Ouchterlony agar-gel diffusion tests depended on the preparation. Figures 1 (Fuller extracts) and 2 (acetone extracts) show the results of precipitation tests performed with undiluted, absorbed serum 4153. As shown in Fig. 1, Fuller extracts yielded very clear precipitation bands. Only one band could be observed between antiserum 4153 and Fuller extract 4153. In comparison, acetone extracts produced a multiplicity of bands. In addition to the clear band seen with the Fuller extract, two or three additional bands appeared. These seemed to be the same in all strains examined.

### Table 1. Agglutination spectrum of A. actinomy cetem-comitans antisera before and after absorption

| Strain | 3349* | 363* | 4153* | 7400* | 64* | 87* |
|--------|-------|-------|-------|-------|-----|-----|
| Unabsorbed | Absorbed | Unabsorbed | Absorbed | Unabsorbed | Absorbed | Unabsorbed | Absorbed | Unabsorbed | Absorbed | Unabsorbed | Absorbed |
| 3349 | 1:160 | 1:160 | 1:80 | - | - | - | 1:20 | - | - | - | - |
| 363 | - | - | 1:640 | 1:320 | - | - | - | 1:40 | - | - | - |
| 4153 | 1:20 | - | 1:40 | - | 1:320 | 1:320 | 1:20 | - | 1:40 | - | 1:40 | - |
| 7400 | - | - | 1:20 | - | - | - | 1:320 | 1:320 | 1:20 | - | 1:640 | 1:320 |
| 64 | - | - | 1:20 | - | - | - | - | - | 1:640 | 1:320 |
| 87 | 1:20 | - | 1:160 | - | 1:20 | - | 1:20 | - | 1:320 | - | 1:640 | 1:320 |

* Absorbed with strains 4153, 87, and 7400.
* Absorbed with strains 3349, 4153, 7400, 64, and 87.
* Absorbed with strains 3349, 7400, and 87.
* Absorbed with strains 3349, 4153, and 87.
* Absorbed with strains 3349, 363, 4153, 7400, and 87.
* Absorbed with strains 3349, 363, 4153, 7400, and 64.
* No agglutination in antiserum diluted 1:20.
Vol. 23, 1972

SEROLOGY OF A. ACTINOMYCETEM-COMITANS

Table 2. Agglutination tests with 100 strains of A.
actinomycetem-comitans using six absorbed
antisera

| No. of strains | Absorbed antisera |
|----------------|-------------------|
|                | 3349 | 363  | 4153 | 7400 | 64  | 87  |
| 23             | A    | -    | -    | -    | -   | -   |
| 1              | -    | A    | -    | -    | -   | -   |
| 9              | -    | -    | A    | -    | -   | -   |
| 1              | -    | -    | -    | A    | -   | -   |
| 1              | -    | -    | -    | -    | A   | -   |
| 23             | -    | -    | -    | -    | -   | A   |
| 2              | A    | A    | -    | -    | -   | -   |
| 2              | A    | A    | -    | -    | -   | -   |
| 6              | A    | -    | A    | A    | -   | -   |
| 4              | A    | -    | A    | -    | -   | -   |
| 3              | A    | A    | -    | -    | -   | -   |
| 3              | A    | -    | A    | -    | -   | -   |
| 1              | A    | A    | -    | A    | -   | -   |
| 1              | A    | A    | A    | -    | A   | -   |
| 1              | A    | A    | A    | -    | -   | A   |
| 2              | A    | A    | A    | -    | -   | -   |
| 4              | -    | A    | -    | -    | A   | -   |
| 1              | -    | -    | A    | -    | A   | -   |
| 4              | -    | -    | -    | A    | -   | A   |
| 1              | -    | -    | -    | -    | -   | A   |
| 1              | -    | -    | -    | -    | -   | A   |
| 54             | 9    | 28   | 9    | 25   | 44  |

* A - agglutination tests positive in antiserum
diluted 1:20 or more; - - no agglutination in anti-
serum diluted 1:20.

In Table 3, the Ouchterlony tests data are
summarized. Unabsorbed antisera and Fuller
extracts of the homologous A. actinomycetem-
comitans strains were used in these studies.
Four of the six extracts yielded only one band
with the corresponding antiserum. Strain 3349
yielded precipitation bands with antiserum 3349
and 363, and strain 87 yielded bands with anti-
sera 87 and 64.

Discussion

A. actinomycetem-comitans is a gram-nega-
tive microaerophilic bacillus normally present
in the human oral cavity. It can be found in
infections, where it usually occurs in company
with Actinomyces israelii, particularly in cases
of human actinomycosis. We have already
demonstrated that A. actinomycetem-comi-
tans has a characteristic fermentation reaction
pattern. Furthermore, on the basis of their fer-
mentative reactions with galactose, mann-
tol, and xylose, the 140 strains studied could
be grouped into eight biotypes (3).

Serological studies with A. actinomycetem-
comitans have thus far been reported only by
Heinrich and Pulverer (1) and by King and
Tatum (2). Heinrich and Pulverer (1) per-
fomed agglutination reactions with 12 A. acti-
nomycetem-comitans strains, and found prac-
tically identical results with antiserum 3349.
Precipitation tests were also done with the
same 12 strains and antiserum 3349 after the
antigens had been prepared by repeated
freezing and thawing. The results of tube pre-
cipitation tests corresponded to the agglutina-
tion tests. King and Tatum (2) examined 33 A.
actinomycetem-comitans strains. Studies with agglutination reactions were abandoned because of spontaneous agglutination. The tube precipitation tests yielded remarkable results: by use of the Lancefield extraction procedure, three different antigens were demonstrated, and named A, B, and C. All 33 strains studied possessed one of these three antigens. No strain possessed more than one antigen.

Our results agree with those of King and Tatum (2), who demonstrated several different antigens in A. actinomycetem-comitans. By studying 100 strains, absorption tests enabled us to establish six antisera. With tube agglutination tests, we showed that 99 of the 100 A. actinomycetem-comitans strains possessed at least one of the six antigens: 3349 [previously tested (1)], 363, 4153, 7400, 64, and 87. Only one strain could not be agglutinated. Most strains possessed only one of the six antigens, but strains with several antigens (up to six) were seen. The most common antigens were antigens 3349 and 87. Antigens 363 and 7400, on the other hand, were observed in only 9% of the 100 strains tested. Altogether, 24 different antigen patterns were found. The results were reproducible and independent of the media used. Therefore, it seems that agglutination reactions can be used for epidemiological and ecological studies on A. actinomycetem-comitans.

Precipitation tests were done with the Ouchterlony agar-gel diffusion method. Antigens were prepared according to the Fuller method and according to the acetone extraction method. Fuller extracts yielded clear results, whereas antigens prepared by the acetone extraction procedure seemed to be less specific because precipitation bands were found which obviously belonged to all strains. The precipitation test is judged inferior to the agglutination test for typing purposes.

LITERATURE CITED

1. Heinrich, S., and G. Pulverer. 1959. Zur Ätiologie und Mikrobiologie der Aktinomykose. II. Definition und praktische Diagnostik des Actinobacillus actinomycetem-comitans. Zentralbl. Bakteriol. Parasitenk. Infektionskr. Hyg. Abt. Orig. 174:123-136.
2. King, E. O., and H. W. Tatum. 1962. Actinobacillus actinomycetemcomitans and Hemophilus aphrophilus. J. Infec. Dis. 111:85-94.
3. Pulverer, G., and H. L. Ko. 1970. Actinobacillus actinomycetem-comitans: fermentative capabilities of 140 strains. Appl. Microbiol. 20:690-695.