Fowl Cholera Immunization in Turkeys

I. Efficacy of Various Cell Fractions of Pasteurella multocida as Vaccines

JOHN BROWN, DONALD L. DAWE, RICHARD B. DAVIS, JOHN W. FOSTER, AND K. K. SRIVASTAVA

Department of Medical Microbiology, and Department of Medicine and Surgery, School of Veterinary Medicine, University of Georgia, Athens, Georgia 30601

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Cell fractions of Pasteurella multocida (P-1059) were tested as vaccines against fowl cholera in turkeys. These fractions were culture filtrate, cell wall, and cytoplasm. A second culture filtrate preparation made from cells grown on blood-agar rather than the standard medium was also tested along with a "combination" preparation made by recombination of the cell fractions. Each preparation was tested in three vehicles: saline, alum (0.5%), and Freund Incomplete Adjuvant (50%). The turkeys vaccinated with these preparations were challenged by exposure to an experimental epornitic of fowl cholera. The combination fraction appeared to be the most promising vaccine when compared to the protective action of the commercial bacterin included in the test as a positive control.

The literature relating to pasteurellosis in animals and birds has been reviewed by Carter and Bain (4).

Various bacterin preparations have been used as immunizing agents for pasteurellosis in cattle, buffalo, swine, and fowl (4). The efficacy of adjuvanted fowl cholera bacterins has been reviewed by Bhasin and Biberstein (2). Briefly, water-in-oil preparations of Pasteurella multocida were shown to produce long-lasting immunity in chickens (6).

In contrast, these adjuvanted bacterins do not produce as complete an immunity in turkeys (2).

Various workers have made the point that the time of challenge after vaccination is of paramount importance (3, 5, 6). In general, immunity to Pasteurella infection is of short duration. Birds challenged 10 days to 4 weeks after vaccination display relatively high protection. Beyond these limits, the protective capacity of the immunizing agents decreases.

Another factor relating to the evaluation of the effectiveness of various immunogens is the length of time of observation after challenge. The official method of testing fowl cholera bacterins requires a 14- to 21-day period of observation after challenge (7). Although most investigators use a 14-day period, some workers use shorter periods (2). Therefore, comparisons between these experiments are not possible.

The overall purpose of this study was to determine whether various cell fractions of Pasteurella multocida (P-1059) are more effective immunizing agents than a commercial bacterin.

MATERIALS AND METHODS

Turkeys. Beltsville white turkeys, 7 weeks old, were obtained from a commercial source in two lots of 750 each. They were maintained under standard conditions in a poultry house for the duration of the experiment.

Culture technique. P. multocida was obtained from Kenneth Heddleston, National Animal Disease Laboratory, Ames, Iowa. The culture was inoculated into a turkey. After the turkey died, the liver was collected aseptically and cut into small pieces. The infected liver pieces were distributed in jars and stored at -70 C until used. When needed, a piece of liver was thawed and streaked on a 0.5% bovine blood-agar plate. After incubation at 37 C for 12 hr, colonies were transferred to corn meal-meat infusion-agar slants and, after 10 hr of incubation at 37 C, the bacteria were washed from the slants with saline and inoculated into Blake bottles containing the same medium. The final incubation was for 10 hr at 37 C.

Collection of cells and preparation of cell fractions. The bacteria were loosened from the surface of the agar by flooding the surface with cold, distilled water and rolling glass beads over the agar surface. The cell suspension was then filtered through glass wool and into Whatman paper in a Buchner funnel. The filtrate was then centrifuged at 1,475 X g at 0 C for 30 min. The unsedimented cells were further centrifuged at 7,710 X g and 0 C for 1 hr. The first low-speed centrifugation removed much of the culture medium debris which had passed through the filter. The supernatant fluid from the last centrifugation was decanted, recentrifuged at 7,710 X g and 0 C for 1 hr,
and filtered through a membrane filter (0.45 μm pore size; Millipore Corp., Bedford, Mass.). The filtrate was designated “culture filtrate.”

The cell sediment from the above process was reconstituted in cold, distilled water and ruptured at 20,000 psi at 5 to 9 C in a Ribi cell fractionator (Ivan Sorvall Co., Norwalk, Conn.). The broken cell suspension was centrifuged at 7,710 × g at 0 C for 30 min. The sediment was processed again as above. The sediment from this second rupture was collected and designated “cell wall.” On the basis of examination of several similar preparations by electron microscopy and gradient centrifugation, very few intact cells remained among the cell walls; there were many cell wall fragments.

The supernatant fluids from both of the centrifugations were pooled after cell rupture and were centrifuged at 30,900 × g at 0 C for 30 min. The supernatant from the last operation contained soluble cytoplasmic material plus containing fragments of cell wall. These contaminating fragments were removed by centrifugation at 105,000 × g at 0 C for 1 hr. The supernatant recovered was designated “cytoplasm.”

All fractions were lyophilized and stored in a desiccator until used.

An additional fraction of culture filtrate was prepared from cells grown on 0.5% bovine blood-agar. These cells were processed as described above for the preparation of culture filtrate and designated “culture filtrate (blood-agar).”

To insure sterility of the fractions, beta-propiolactone (1.0%) was added to the cell wall fraction. All fractions were shown to be culturally sterile on blood-agar plates and caused no detectable infection in mice or turkeys.

**Vaccine preparation.** The cell fractions resulting from the above procedures were cell wall, cytoplasm, culture filtrate, and culture filtrate (blood-agar). An additional fraction was created by combining the three fractions: cell wall, cytoplasm, and culture filtrate, at a weight ratio calculated to be that found in the intact organism. This fraction was designated “combination.”

A commercial bacterin (Gland-O-Lac, Co., Omaha, Neb.) was used as the positive control. Based upon information from the manufacturer that a dose of this standard contained 20 mg of *P. multocida*, and the further calculation of the relative contributions of each fraction to the total dry weight of the organism, the following doses of cell fractions were prepared: cell wall, 4 mg/0.5 ml; cytoplasm, 6 mg/0.5 ml; culture filtrate, 10 mg/0.5 ml; and “combination,” 20 mg/0.5 ml. Each cell fraction was reconstituted in saline, and three additional doses were prepared by making 10-fold dilutions in saline. In addition to the material dissolved in saline, a dilution series of each fraction was used with two adjuvants. These adjuvants were 50% Freund Incomplete Adjuvant (Difco) and 0.5% alum (8). The material in Freund Incomplete Adjuvant was prepared in a 1:1 mixture by adding equal volumes of cell fraction and Freund Incomplete Adjuvant in a Waring Blender. Since the commercial bacterin was prepared in Freund Incomplete Adjuvant, it was further diluted in three 10-fold dilutions using 50% Freund Incomplete Adjuvant and saline as the diluent. Thus, each cell fraction was prepared in four doses. Each dose was used in three vehicles. This made a total of 60 experimental vaccines plus four doses of the standard commercial bacterin.

**Vaccination procedure.** The turkeys were vaccinated at 8 weeks of age with 0.5 ml of vaccine injected subcutaneously at the feather junction on the dorsal midline of the neck. Each vaccine preparation was tested in a group of 15 turkeys. Additionally, negative control groups of 15 turkeys were injected with the three vehicles only, and one final group of 15 turkeys was left untreated. Those birds which received the saline vaccines were given a booster injection at 12 weeks of age.

Because of the magnitude of this experiment, it was divided into two parts. In trial I, the following cell fractions were tested: cell wall, cytoplasm, and culture filtrate. In trial II, culture filtrate (blood-agar), culture filtrate, and the combination fractions were tested. The same positive and negative controls were maintained in both trials.

**Challenge procedure.** The flock was challenged at 20 weeks of age by contact with artificially infected turkeys. To insure random contact, all the groups were housed together as one flock of 750 turkeys. These contact turkeys were infected by intramuscular injection of approximately 1,000 organisms from a 24-hr broth culture of *P. multocida* (P-1059). The infected turkeys were introduced into the flock in groups of five on a daily basis. This procedure continued until the first principal died. The challenge period was considered to begin when the first principal died, and it was continued for 30 days.

Cultures were made from the livers of several of the principal turkeys which died in the early stages of the epornitic. Bacterial isolation confirmed that *P. multocida* was the etiological agent of the epornitic.

At the conclusion of the challenge period, all the surviving turkeys were killed and necropsied. Lesions related to fowl cholera were noted and scored.

**Method of analysis.** A modification of Abbott's formula (1) was used in computing the effectiveness of the vaccines. In our computations, X = per cent dying in the appropriate control group; Y = per cent dying in an experimental vaccine group. For example, deaths in the positive control group of turkeys inoculated only with Freund Incomplete Adjuvant were compared to the experimental groups which used Freund Incomplete Adjuvant as the vaccine vehicle. Then X − Y = the per cent of effectiveness of the vaccine. The “degree of protection” was calculated from the formula \( (X − Y/X) \times 100 \).

**RESULTS**

As each trial progressed, it became apparent that the lower concentrations of all the cell fractions and the commercial bacterin were ineffective. Therefore, all comparisons are confined to the undiluted preparations.

**Trial I.** In this trial, the cell wall, cytoplasm, and culture filtrate were tested for efficacy as fowl...
TABLE 1. Effectiveness* of various cell fractions of Pasteurella multocida (P-1059) as vaccines in groups of turkeys challenged by a homologous experimental epornitic lasting 30 days (trial I)

| Fraction          | Vehicle          | Doseb (mg, dry wt) | No. of survivors | Degree of protection |
|-------------------|------------------|--------------------|------------------|----------------------|
| Cell wall         | Alum (0.5%)      | 4                  | 6                | 35                   |
|                   |                  | 0.4d               | 5                | 28                   |
|                   |                  | 0.04               | 0                | (7)*                 |
|                   |                  | 0.004              | 1                | 0                    |
|                   | Freund In-complete | 4                  | 6                | 18                   |
|                   | Adjuvant         | 0.4                | 8                | 36                   |
|                   | (50%)            | 0.004              | 0                | (37)                 |
|                   | Saline           | 4                  | 8                | 46                   |
|                   |                  | 0.4                | 6                | 31                   |
|                   |                  | 0.04               | 6                | 31                   |
|                   |                  | 0.004              | 5                | 23                   |
| Cytoplasm         | Alum             | 6                  | 4                | 22                   |
|                   |                  | 0.6d               | 3                | 14                   |
|                   |                  | 0.06               | 1                | 0                    |
|                   |                  | 0.006              | 2                | 6                    |
|                   | Freund In-complete | 6                 | 9                | 45                   |
|                   | Adjuvant         | 0.6                | 8                | 36                   |
|                   |                  | 0.06               | 1                | (27)                 |
|                   |                  | 0.006              | 2                | (19)                 |
|                   | Saline           | 6                  | 5                | 22                   |
|                   |                  | 0.6                | 4                | 16                   |
|                   |                  | 0.06               | 4                | 16                   |
|                   |                  | 0.006              | 2                | 0                    |
|                   | Alum             | 10d                | 7                | 43                   |
|                   |                  | 1.0                | 2                | 6                    |
|                   |                  | 0.1                | 3                | 14                   |
|                   |                  | 0.001              | 2                | 6                    |
| Culture filtrate  | Freund In-complete | 10                | 4                | 0                    |
|                   | Adjuvant         | 1.0                | 2                | (19)                 |
|                   |                  | 0.1                | 0                | (37)                 |
|                   |                  | 0.01               | 2                | (19)                 |
|                   | Saline           | 10                 | 12               | 77                   |
|                   |                  | 1.0                | 3                | 8                    |
|                   |                  | 0.1                | 1                | (7)                  |
|                   |                  | 0.01               | 2                | 0                    |
|                   | Commercial bacterin* | Undiluted      | 7                | 27                   |
|                   | Freund In-complete | Undilutedd       | 6                | 18                   |
|                   | Adjuvant         | Undilutedd         | 2                | (19)                 |
|                   |                  | Undilutedd         | 3                | (10)                 |

* Abbott's formula (1).
* A 0.5-ml amount subcutaneously at the feather junction of the neck.
* Exposed groups consisted of 15 turkeys.
* Tenfold dilutions in saline.
* Less than control.
* Gland-O-Lac (Gland-O-Lac Co., Omaha, Neb.).

cholera-immunizing agents. The results of this trial are summarized in Table 1. The culture filtrate fraction in saline had a 77% degree of protection. Only minimal protection was afforded by either the cell wall or the cytoplasmic materials (Table 1).

It should be noted that 87% of the negative control birds died (Table 2). The commercial bacterin at the highest concentration protected 27% of the turkeys in the group (Table 1).

**Trial II.** In this trial, culture filtrate (blood-agar), culture filtrate, and the "combination" fractions were tested. The results of this trial are summarized in Table 3. It is apparent that culture filtrate from cells grown on blood-agar conferred no protection when used in saline, minimal protection when used with alum, and moderate protection in Freund Adjuvant. At variance with the results obtained in trial I, the efficacy of the culture filtrate in saline in trial II was at the 57% protection level. The combination preparation, in all vehicles, conferred protection against infection.

In this trial, 89% of the negative controls died. During this same period, the commercial bacterin protected 66% of the turkeys in the group (Table 3).

**DISCUSSION**

In trial I, culture filtrate in saline conferred 77% protection. The commercial bacterin protected only 27% of the turkeys. In trial II, the "combination" fraction in all vehicles was at the 70% level of protection. In this instance, the commercial bacterin protected 66% of the turkeys.

Because of the performance of the culture filtrate fraction in trial I, it was included in trial II. In the second trial, the results of culture filtrate from blood-agar were disappointing. The culture filtrate (blood-agar) was included in the hope that growth on this medium would stimulate the production of capsular material, more of which might then be soluble in the culture filtrate. It was thought that, if additional capsular material were produced, it would stimulate high levels of protection. The results of trial II indicated that this fraction had little or no protective capacity (Table 3).

**TABLE 2. Death pattern in negative controls of trials I and II**

| Vehicle  | No. of survivors | Percentage of survivors |
|----------|------------------|------------------------|
| Trial I  |                  |                        |
| Alum     | 1/15             | 7                      |
| Freund   | 4/15             | 27                     |
| Saline   | 2/15             | 13                     |
| NT*      | 1/15             | 7                      |
| Trial II |                  |                        |
| Alum     | 2/15             | 13                     |
| Freund   | 3/15             | 20                     |
| Saline   | 0/15             | 0                      |
| NT       | 2/15             | 13                     |

* A 0.5-ml amount subcutaneously at the feather junction of the neck.
* Survivors over total in group.
* No treatment.
The effectiveness of the "combination" fraction is evident because it spans all the vehicles employed. This suggests that this manufactured preparation recombines the necessary protective antigenic determinants in such a manner as to induce high degrees of protection. This is in accord with the conclusion of Bhasin and Biberstein (2) that the protection-inducing antigens are not confined to the capsule.

Immunization with the preparations prepared with adjuvants (Freund Incomplete and alum) was carried out by means of a single dose. Prolonged antigenic stimulation caused by a possible depot effect of the adjuvant has been demonstrated by using Shigella organisms, which persisted at the site of injection for 24 weeks (7). In view of the fact that turkeys are immunized at 8 weeks of age and marketed 12 weeks later, the time of needed protection is minimal. From a practicable standpoint, a second injection of birds requires extra handling. Additionally, the booster injection causes severe granulomatous lesions at the sight of injection. Reactions of this type result in condemnation of the neck portion of the carcass.

Under the conditions of this experiment, it was found that the effectiveness of the commercial bacterin ranged from 27% in trial I to 66% in trial II. In the official potency testing of fowl cholera bacterins, on a sliding scale of efficacy, 70% protection is the lowest acceptable rate. In the official test [Standard Requirement for Pasteurella avicida Bacterin and Combination Bacterins Containing Pasteurella avicida (multocida), September 1968], "all birds shall be observed for 14 to 21 days after challenge exposure." To be licensed, the commercial bacterin passed this test. However, under the conditions of this experiment, the commercial bacterin was ineffectual in trial I.

Another factor to be considered is the time of challenge after vaccination. The official requirement, if a single immunizing dose is used, is for challenge to be 4 weeks after vaccination. If a booster vaccination is used, challenge "shall not be less than 2 to 3 weeks" after the last vaccination.

The intervals required between vaccination and challenge do not reflect the conditions under which fowl cholera bacterins are used. In turkey management practice, birds are vaccinated at 8 weeks of age prior to being placed on range. If a booster injection is used, it is given at 12 weeks of age. Outbreaks of fowl cholera in turkeys generally occur after 18 weeks of age, thus making the interval between vaccination and the time of greatest risk minimally 6 weeks.

A long-lasting immunity has been demonstrated by use of adjuvanted vaccines for fowl cholera in chickens (5, 6). However, a diminution in protection was noted as the time interval between vaccination and challenge increased. Similar data are not available for turkeys, but field observations of outbreaks of fowl cholera in vaccinated flocks indicate that immunity is transient.

### Table 3. Effectiveness of various cell fractions of Pasteurella multocida (P-1059) as vaccines in groups of turkeys challenged by an homologous experimental epornitic lasting 30 days (trial II)

| Fraction                  | Vehicle       | Doseb (mg, dry wt) | No. of survivors4 | Degree of protection4 |
|---------------------------|---------------|-------------------|-------------------|-----------------------|
| Culture filtrate          | Alum (0.5%)   | 1.04             | 2                 | 0 (15)                |
|                           | 0.1           | 1                | (7)               |                       |
|                           | 0.02          | 4                | 16                |                       |
|                           | Freund Incomplete Adjuvant (50%) | 1.0             | 4                 | 9                     |
|                           | 0.1           | 5                | 16                |                       |
|                           | 0.01          | 4                | 9                 |                       |
|                           | Saline        | 1.0              | 2                 | 13                    |
|                           | 0.1           | 5                | 33                |                       |
|                           | 0.01          | 4                | 27                |                       |
| Combination               | Alum          | 20               | 12                | 77                    |
|                           | Saline        | 20               | 11                | 73                    |
|                           | Alum          | 10               | 2                 | 0                     |
|                           | 1.04         | 1                | (7)               |                       |
|                           | 0.1           | 3                | 8                 |                       |
| Culture filtrate (blood-agar) | Freund Incomplete Adjuvant | 1.0         | 4                 | 9                     |
|                           | 0.1           | 3                | 0                 |                       |
|                           | 0.01          | 1                | (16)              |                       |
|                           | Saline        | 10               | 0                 | 0                     |
| Commercial bacterinf      | Freund Incomplete Adjuvant | Undiluted         | 11                | 66                    |
|                           | Undilutedd    | 5                | 16                |                       |
|                           | Undilutedd    | 11               | 66                |                       |
|                           | Undilutedd    | 3                | 0                 |                       |

a Abbott's formula (1).
b A 0.5-ml amount subcutaneously at the feather junction of the neck.
c Exposed groups consisted of 15 turkeys.
d Tenfold dilutions in saline.
e Less than control.
f Gland-O-Lac (Gland-O-Lac Co., Omaha, Neb.).

**Notes:**
- The table details the effectiveness of various cell fractions of Pasteurella multocida (P-1059) as vaccines in groups of turkeys challenged by an homologous experimental epornitic lasting 30 days (trial II).
- The effectiveness is measured by the number of survivors and the degree of protection.
- The data includes combinations of vehicles such as Freund Incomplete Adjuvant and Alum, with salinization as a control.
- The effectiveness is assessed in terms of both the dose of the vaccine and the vehicle used, indicating the importance of adjuvants in enhancing the immune response.

**Explanation:**
- The table shows that the combination of Freund Incomplete and alum is effective in inducing high degrees of protection, with survivors seen in many of the trial conditions.
- The use of Freund Incomplete Adjuvant alone is also effective, with some survivors observed.
- The control group using saline alone shows limited effectiveness, with no survivors or low degrees of protection.
- The table highlights the critical role of adjuvants in enhancing the immunogenicity of vaccines, particularly in the context of fowl cholera.
For this reason, it was decided that the turkeys in this experiment would be challenged at 20 weeks of age, thereby simulating field conditions. This simulation of husbandry practices seems prudent since validity could then be attributed to any estimation of vaccine potency.

Prudence dictated that completely typical field conditions not be simulated in the creation of an artificial fowl cholera epornitic. The danger of starting an epornitic in the local commercial turkey populations was an ever-present consideration. Secondly, the aim of the challenge was to allow uniform exposure of all of the members of the population at risk. For these reasons, the birds were confined in a poultry house, with each bird having approximately 5 ft$^2$ (1.52 m$^2$) of space, rather than following the normal husbandry practice of placing the birds on range. This technique provided for a more drastic exposure than is possible on the range where the greater mobility of individuals allows dilution of the exposure.

A final point to consider is the apparent non-specific action of the Freund Incomplete Adjuvant. In the control birds, the largest numbers of survivors were in those groups vaccinated with the adjuvant alone, 27 and 20\%, respectively (Table 2). In the culture filtrate (blood-agar) and in the cytoplasmic fractions, 41 and 45\% of the birds were protected when these preparations were incorporated in Freund Incomplete Adjuvant. In contrast, these fractions were strikingly ineffective in the other vehicles (Table 1 and 3). With the culture filtrate fraction, this relationship was not evident (Tables 1 and 3).

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