STUDIES ON *PASTEURELLA MULTOCIDA*

IV. THE IMMUNIZING VALUE OF EGG AGAR VACCINE AGAINST FOWL CHOLERA

Eihyo RYU

*Department of Veterinary Medicine, College of Agriculture,
National Taiwan University, Taipei, Taiwan, China*

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Many preparation methods for pasteurella vaccine, used for controlling pasteurellosis or hemorrhagic septicemia, have been reported. These reported vaccines can be classified into the following nine kinds: Live vaccine (VIANELLO¹, HUDSON², OSMETTI³, THAL⁴, VAN DORSSEN⁵), Heated vaccine (QUEEN and QUORTRUP⁶, SINHA et al.⁷), Formolized vaccine (GENEIDY⁸, CARTER⁹), Chromo vaccine (REMLINGER¹⁰, HUG and QADIR¹¹), Saponin vaccine (GAZARYAN and POGOSYAN^{12, 13}, AIRAPETYAN¹⁴, LEKVEISHVILI¹⁵), Chicken embryo vaccine (CATER¹⁶, YODER and MERCHANT¹⁷, PETEK et al.¹⁸), Aluminum hydroxide-gel vaccine (SUHACI et al.¹⁹, GRIGORIU et al.²⁰), Alum precipitated vaccine (NIKIFOROVA^{21, 22}) and Oil adjuvant vaccine (BAIN^{23, 24}, VANCHESWARA et al.²⁵, BAIN and JONE²⁶, DHANDA et al.²⁷). Each of the above vaccines were reported to have satisfactory results by the workers using them, but a large scale comparison test, relating to these vaccines, has not been made, although limited experiments have been made by the following authors: LOUSTAU²⁸ on the comparison of several preparation of fowl cholera vaccine, recommended formolized (0.5%) serum broth culture for the prevention of fowl cholera, because living organisms were generally of little value. In his report, he also described that phenolized serum broth culture was equally effective against the infection per os, but it was only half as protective against intramuscular infection. CARTER²⁹ has shown that the chicken embryo vaccine protected mice against more than 1,700 lethal doses of *Past. septica*, but the protection conferred by broth bacterin was, at most, not in excess of six l.d. of the same challenge culture. DOUGHERTY³⁰ had used *Past. septica*, isolated from ducks, to prepare broth bacterins and duck embryo formolized vaccine for the control of fowl cholera in Peking ducks, and then compared them with four other commercial bacterins, and reported that the duck embryo vaccine gave much better protection than any of the commercial bacterins and that it protected 83% of the ducklings 4 weeks after immunization. SHIRLAWS³¹ prepared a formolized vaccine from strains of high virulence and rich capsule content to confer a high level immunity on cattle and laboratory animals, but embryo vaccine, immune serum, aggrassin, filtrate of broth culture, and saponin vaccine, were proved of little or no value. On the other hand, a high degree of immunity was obtained by a vaccine prepared from a rough, avirulent Kenya strain of *Past. bovis septica*.

As the above literature shows, the reason why the same kind of vaccine had unequal effect is probably due to the difference in types of strains, virulence, laboratory animals, and the preparation methods for the vaccine.

Fowl cholera and swine pneumonia have been regarded as the two main kinds of pasteurellosis found in our country, and commercial vaccines have not yet given any sufficient immunity.

In a previous report, I have reported that blood, as well as serum, would not be

suitable for enriching the medium, and that the whole egg agar would give a superior result for the growth of *Past. multocida*. In attempting to produce a strong immunity we, in this experiment, began to use egg agar as the culture medium for the preparation of vaccine, and then we used the egg agar vaccine to immunize fowls and mice in order to compare its result with that of chicken embryo vaccine, commercial vaccine, and broth culture. I herein report the results as follows.

MATERIALS AND METHODS

1. The preparation methods for vaccines

Although the main purpose of this study is to determine the protective value of egg agar vaccine, it was also necessary to prepare other vaccines for comparison with the egg agar vaccine. These vaccines were, aluminum hydroxide gel (AHG) egg agar vaccine, chick embryo vaccine, AHG chicken embryo vaccine, and formolized broth culture. The preparation methods for these vaccines are shown as follows.

a) Egg agar vaccine

The egg agar medium was used as the culture medium for the preparation of this vaccine. To prepare egg agar medium, the nutrient agar was previously adjusted to pH 7.4 and then tubed. After sterilization, the content of a whole fresh leghorn egg was added to each of the tubes, so that the concentration was 10% of the medium. It was then mixed well to make a slant. To prepare the vaccine, a loop of broth culture under cultivation for 20–24 hours, from F5 strain, was diluted in 5 cc of saline solution, and then 0.3 cc of this suspension was put into the egg agar slant. After the surface of the slant was equally moistened by the added suspension, it was placed in the incubator for cultivation for as long as 24 hours. After the growth was completed, 10 cc of saline solution was put into each of the cultures, and they were then dissolved by a whirl loop and placed in a bottle which contained glass balls. Formalin was added up to the concentration of 0.2%, and the preparation was then kept at room temperature for 48 hours. These vaccines contained 6–16 billion organisms per 1 cc.

b) Aluminum gel egg agar vaccine

It was prepared from mixing an equal volume of egg agar vaccine with aluminum hydroxide gel. The gel was made by adding 5% ammonia solution to 25% ferric aluminum solution, and was then washed in distilled water and sterilized.

c) Chicken embryo vaccine

After inoculating 0.1 cc of broth culture of F5 strain into a 7-day-old leghorn embryo, the embryo was incubated for 48 hours in the incubator. Afterwards the embryo content was removed and saline solution was added to it in the ratio of 1/10 and the whole emulsified. Then sufficient formalin was added to the preparation to give a final concentration of 0.24 percent.

d) AHG chicken embryo vaccine

It was prepared by mixing equal volumes of chicken embryo vaccine and AHG.

e) Formolized broth culture

The formolized broth culture was prepared by adding 10% formalin into the 24 hour broth culture of F5 strain to make a concentration of 0.2 percent.

2. Strain

The *Past. multocida* which were used in the preparation of vaccine and challenge was of strain F5. This strain was one of high virulence and had been isolated from a fowl affected by fowl cholera, and had then been subcultured on media for more than one year. This cultures could cause a white leghorn of 1–3 months of age to sicken with fowl

cholera through the oral administration of 1 cc. It also killed fowls through the intramuscular administration of a 10^{-4} dilution of broth culture. It gave to mice $10^{-7.74}$ of LD50, and was demonstrated to possess a large capsule when grown on the egg agar medium.

3. Experiment animals

The DD strain of white mice, 12–15 grams in weight, and white leghorns, 2–3 months of age, were used for the immunity experiments. The white leghorns had been grown quickly in batteries and had reached 1.0 kg in body weight when we began to use them.

4. Immunization and challenge

The inoculating route for the vaccine was either intraperitoneal or subcutaneous in mice, and intravenous or intramuscular in fowls, while the challenge route was subcutaneous in mice, and intramuscular on the opposite side from the inoculation, in fowls. The inoculation doses of vaccine were 0.1 cc to each of the mice, and to the fowls they were given as shown in the Table. Challenge followed 2 weeks after immunization.

EXPERIMENTS

Experiment 1. The toxicity of egg agar vaccine administered into blood vessels

Formerly, NAKAMURA³²⁾, in his study on chicken embryo vaccine from Newcastle disease virus, reported that the vaccine could confer sufficient immunity if administered intravenously, but it would not be effective enough when administered intramuscularly.

As pasteurellosis is a septicemia, it seems to be ideal for giving sufficient immunity when the vaccine is induced into the blood vessel, and so we began to prepare several kinds of egg agar vaccine. In order to compare the immunizing values of different vaccines which were prepared from different chemicals, the following chemical preparations such as 0.5% Na_2CO_3 , 0.5% phenol, 0.1–0.2% formalin, were added to the vaccine. Heated vaccine, inactivated at 60–80°C, was also prepared. Comparative tests among these vaccines were then carried out two times by injecting them into the blood vessels of fowls.

It resulted, in this experiment, that independent of the kind of vaccine used, fowls showed dullness, and lack of appetite, and some died within a night, or within 1–2 days. The toxicity of the vaccine is given in Table 1.

As shown in Table 1, these vaccines, inactivated with 0.5% Na_2CO_3 , 0.5% phenol,

Table 1. Comparison of Toxicity of Egg Agar Vaccine by Intravenous Injection with Fowls

Vaccine inactivated with	No. of experiments	Inoculated dose	Survive number
0.5% Na_2CO_3	1 2	1.0cc 0.5	1/5* 4/5
0.5% Phenol	1 2	1.0 0.5	4/5 3/5
Formalin	1 (0.2%) 2 (0.1%)	1.0 0.5	5/5 3/5**
Heat	1 (60°C 30 min.) 2 (80°C 30 min.)	1.0 0.5	2/5 4/5

* Numerator equals number of survivors: denominator equals number of fowls used.

** *Past. multocida* was isolated from carcasses.

and heat, were proved to be highly toxic in the two experiments, and each of them resulted in postvaccinal losses. On the contrary, fowls vaccinated with 0.2% formalized egg agar vaccine (0.2%) were all resistant, but vaccinal losses, however, occurred in the group of fowls vaccinated with 0.1% formalized egg agar vaccine on account of insufficient inactivation with formalin. From the fact that the heat vaccine had shown a 3/5-1/5 loss, it is clearly understood that the losses through vaccination were not due to the chemicals, but may be suspected to have been due to the toxicity of the capsule of pasteurilla organisms. In the two experiments, formalized vaccine (0.2%) has never resulted in any vaccinal losses.

Experiment 2. The effect of egg agar vaccine administrated intravenously

Next, I began to use formalin killed vaccine (0.2%) which contained 12 billions of the organisms per 1 cc vaccine for the immunization of 20 fowls by the intravenous route. 1-2 weeks later, they were challenged, intramuscularly, with 1 cc of 10^{-4} of the F5 strain broth culture. The values of the immunizing effects are given in Table 2.

As shown in Table 2, the immunizing effect was but little different 1 week and 2 weeks after immunization, and showed as high immunity as 77.8% survival when the egg agar vaccine was induced into the blood vessel. However, it was an unexpected result

Table 2. Immunizing Value of Egg Agar Vaccine by Intravenous Injection with Fowls

Immune period	Survive number	Control
7 day	7/9	0/10
14	7/9	2/10
Numerator equals number of survivors: denominator equals number of fowls used.		

Table 3. Comparison of Immunizing Value of Formalized (0.2%) Egg Agar Vaccine by Intraperitoneal and Subcutaneous Injection with Mice

Dilution	Route of Immunization		Control
	Subcutaneous	Intraperitoneal	
10^{-5}	4/5*	3/5	0/5
10^{-6}	5/5	3/5	0/5
10^{-7}	5/5	5/5	0/5
10^{-8}	5/5	5/5	4/5
10^{-9}	5/5	5/5	4/5
L.D.50	$<10^{-5}$	$10^{-5.22}$	$10^{-7.74}$

* Numerator equals number of survivors: denominator equals number of mice used.

Table 4. Comparison of Immunizing Value of Egg Agar Vaccine by Intramuscular Injection with Fowls

Experiment	Preparation	Dose	Number of bacteria billion/cc	Survival number
I	Egg agar vaccine	1cc	16.8	8/10
	AHG egg agar vaccine	2cc	8.4	9/10
	Chicken embryo vaccine	1cc		3/10
	AHG chicken embryo vaccine	2cc		5/10
	Control			0/10
II	Egg agar vaccine (fresh)	1cc	6.3	6/10
	Egg agar vaccine (fresh)	2cc	6.3	6/10
	Egg agar vaccine (two months)	1cc	16.8	6/10
	Commercial vaccine	2cc		5/10
	Formolized broth culture	2cc		4/10
	Control			0/10

that 2 out of the 20 tested fowls were postvaccinal losses.

Experiment 3. Immunization test of mice

In the previous experiment, there were many losses when the vaccination was by the intravenous route, so that it was necessary to consider other ways such as the intramuscular or subcutaneous routes, for vaccination. Previously, I used mice for this purpose, and immunized them by the intramuscular or subcutaneous routes with 0.1 cc of vaccine (9.4 billion/cc). Two weeks after immunization, the mice were challenged with F5 strain culture. The results are given in Table 3.

As shown in Table 3, the difference between the immunizing effects of the intraperitoneal and subcutaneous routes were not so different; the LD50 in the subcutaneous route was $<10^{-5}$, and $10^{-5.22}$ in the intraperitoneal route. The LD50 of the control was $10^{-7.74}$.

Experiment 4. The immunization of fowls by intramuscular route

The following tests were carried out expecting that the results of the immunization of fowls by the intramuscular route might be similar to that in the case of mice. In the first experiment, four kinds of vaccine, such as egg agar vaccine, AHG egg agar vaccine, chicken embryo vaccine, and AHG chicken embryo vaccine, were compared with respect to the effect of the immunization. In the second experiment, newly prepared egg agar vaccine, and a two-month-old preserved egg agar vaccine, were compared with commercial vaccines, and formolized broth culture. The doses of each vaccine were one to two cc, and the fowls undergoing immunization were challenged with 10^{-4} of the strain F5 2 weeks after the vaccination, in each experiment. The results of the above two experiments are given in Table 4.

As shown in Table 4, the effect of egg agar vaccine, in the first experiment, was that the degree of immunity was higher than that of the chicken embryo vaccine. Moreover, it was very interesting that the addition of AHG increased the immunizing effect of both vaccines, especially in the chicken embryo vaccine. In the second experiment, it was found that there was no influence due to the preservation of the vaccine (fresh or two months) or the amount of the vaccinating dose (1 cc or 2 cc). Each of them showed a stronger immunizing effect than did the commercial vaccines or formolized broth culture.

As mentioned above, egg agar vaccine might give high immunity, but in these experiments, the minimum number of bacteria for producing high immunity was not determined. It is a superior character of the egg agar vaccine that the bacterial number in the vaccine are more easily increased or decreased than in the case of chicken embryo vaccine or broth culture.

CONCLUSION

The author prepared formolized (0.2%) egg agar vaccine from nutrient agar added whole egg, and then compared its immunizing effects with those of AHG egg agar vaccine, chicken embryo vaccine, AHG chicken embryo vaccine, commercial vaccine, and formolized broth culture. The results obtained are as follows.

(1) Fowls immunized with egg agar vaccine by the intravenous route, had shown resistance against the challenge with 10^{-4} cc of broth culture and resulted in 7/9-7/9 of survival (77.8%) in one to two weeks after immunization. However, one should avoid inoculating the vaccine into the blood vessels, as the vaccine is toxic.

(2) Mice immunized with formolized egg agar vaccine by the subcutaneous and intraperitoneal routes had shown $<10^{-5}$ and $10^{-5.22}$ of LD50, the control was $10^{-7.74}$ of LD.

(3) Fowls immunized with formolized egg agar vaccine by the intramuscular route showed 6/10-8/10 of survival (60-80%), this immunizing effect was superior to that of the

chicken embryo vaccine and formolized broth culture when compared with egg agar vaccine.

(4) The immunizing value of chicken embryo vaccine was inferior to AHG chicken embryo vaccine.

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Pasteurella multocida に関する研究

IV. 家禽コレラに対する鶏卵寒天ワクチンの免疫価値

劉 栄 標

国立台湾大学農学院獣医学系細菌学研究室

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著者は前報の鶏卵寒天を用いて製造したフォルマリン (0.2%) ワクチンを以て, 1kg 前後の白色レグホン鶏に注射し, その免疫効果を AHG 加鶏卵寒天ワクチン, 鶏卵 (胚胎) ワクチン, AHG 加鶏卵ワクチン, 市販ワクチン及びフォルマリン・ブイヨン培養と比較して次の如き成績を得た.

(1) 鶏卵寒天ワクチンを鶏翼下静脈に注入し, 1-2 週後に強毒菌株 F5 の 10^{-4} を以て攻撃すれば 7/9-7/9 (77.8%) の生存率を示した. 然し免疫鶏はワクチンの毒性による注射損失を出すので, 実地に之を応用することは出来ない.

(2) 鶏卵寒天ワクチンによる免疫マウスの F5 株攻撃に対する防禦価は皮下免疫では $LD < 10^{-5}$, 腹腔内免疫では $LD 10^{-6.22}$ なるに対し, 対照は

$LD 10^{-7.74}$ を示した.

(3) 鶏を用いて行った免疫試験に於て, 鶏卵寒天ワクチン注射後 2 週間後に F5 株ブイヨン培養の $10^{-4}cc$ を以て攻撃を行った場合の生存率は 6/10 から 8/10 の間にあり, 之の成績は明らかに鶏卵ワクチンやフォルマリン・ブイヨン培養に比して優秀な免疫効果を示した.

(4) 鶏卵ワクチンは AHG を加えた場合その免疫産性能は, 之を加えないものに優る.

以上之を要するに, 鶏卵寒天培地は *Pasteurella* に対する発育が良く, 此の培地を用いて製造したフォルマリンワクチンは 1cc 中に 170 億近く迄菌数を高めることが出来るので, 従来鶏卵ワクチンよりも優良な免疫性能を賦与することが出来る.