STUDIES ON THE ETIOLOGY OF INFECTIOUS ATROPHIC RHINITIS OF SWINE

IV. AGGLUTINATION TEST WITH FORMOLIZED ANTIGEN FOR *BORDETELLA BRONCHISEPTICA* INFECTION IN PIGS

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(Received for Publication August 19, 1972)

In their previous investigation¹⁾, the authors devised an agglutination test procedure, "bouillon method", for diagnosis of *Bordetella bronchiseptica* infection in pigs.

In this method, however, the use of living organisms in broth culture may be inconvenient for field practice. Therefore, it is necessary to devise some effective procedures using killed organisms or non-infective antigens.

The experiments described here were carried out to compare agglutinability between living and formolized antigens with sera from naturally or artificially infected pigs in consideration of test strains, culture media, antigen preparation, reaction temperatures, and reactivity with anti-K, H and O sera.

MATERIALS AND METHODS

1. Strains: Seventeen strains of *B. bronchiseptica* isolated from nasal cavities of naturally infected pigs and maintained on blood agar were used. Ten of them, S-13, S-15, S-19, S-21, H-1, H-4, H-16, H-18, H-20 and H-24, were of only a few subcultures after isolation, while others of 10, or more subcultures. Strain S-19, originated from the nasal cavity of a naturally infected pig, was most frequently used in this study.

2. Culture media: The constituents of the media used for the preservation of the stock culture and antigen preparation are shown in Table 1. A preservative medium was prepared by the method of Ross et al.⁶⁾ with a slight modification.

3. Antigen preparation: Different colonies in appearance were fished and subcultured 1 to 3 times on blood agar plate to confirm the stability of respective colonial features. And, fairly stable phase I colonies were selected from growth on blood agar and used for antigen preparations. All the antigens were prepared from 18 to 20 hour cultures at 37°C. The following four kinds of antigens were prepared: (a) living organisms grown on tryptose broth, nutrient agar, blood agar and charcoal agar, (b) 0.1% formalin-killed organisms from charcoal agar, (c) 0.05% merthiolate-killed organisms from charcoal agar and (d) organisms from charcoal agar heated for 1 hour at 60°C or 100°C and those for 10 minutes at 120°C. The concentration of antigens from the cultures on solid media was adjusted to McFarland's nephelometer No. 5 with phosphate buffered saline, pH 7.2 (PBS) before use.

4. Antisera: Anti-K, H and O sera were prepared in the same manner as described by KASUGA et al.³⁾ and NAKASE⁵⁾. Naturally or artificially infected pig sera were collected at our laboratory, and hysterectomy-produced and colostrum-deprived (HPCD) pigs sera

Table 1. Constituents of Culture Media

1. Blood agar plate medium :
Trypto-Soy Agar Medium (Eiken)
Horse blood, 5 to 10%
2. Trypto-soy broth :
Trypto-Soy Broth (EiAen)
3. Nutrient agar plate medium :
Nutrient Agar (Eiken)
4. Charcoal agar plate medium :
Casamino acid 10 g
Polypeptone 5 g
Yeast extract 2 g
Charcoal 5 g
Agar 20 g
Sodium chloride 5 g
Distilled water 1,000 ml
5. Tryptose phosphate semisolid medium :
Tryptose Phosphate Broth (Difco) +
5% calf serum + 0.2 % agar

were kindly supplied by Dr. NAMIOKA of the National Institute of Animal Health, Tokyo. Sera from conventional *B. bronchiseptica*-free pigs served as controls.

5. Agglutination test: The tube agglutination was used throughout this work. Antisera were diluted to 1 : 5 with PBS, and then heated at 56°C for 30 minutes. To 0.5 ml amount of each serial twofold dilution was added an equal amount of antigens. After shaking, the mixtures were incubated at 37°C or 56°C for 2 hours and then kept in a refrigerator at 4°C overnight.

The result was judged as positive when definite granules of antigen were seen in a

Table 2. Agglutinability of Different Strains of *B. bronchiseptica* Organisms

Strains	Immune rabbit serum						Artificially infected pig serum		
	No. 3 anti-K	No. 4 anti-K	No. 1 anti-H	No. 2 anti-H	No. 5 anti-O	No. 6 anti-O	B-2 anti-K	B-5 anti-K	B-7 anti-K
S- 13	40960	40960	<80	160	<80	<80	1280	640	320
S- 15	40960	20480	<80	80	<80	<80	640	320	160
S- 19	40960	40960	<80	160	<80	<80	640	640	320
S- 21	40960	20480	<80	80	<80	<80	640	320	160
H- 1	160	160	>2560	>2560	1280	2560	80	80	40
H- 4	20480	20480	<80	80	<80	<80	640	640	320
H- 16	40960	40960	<80	80	<80	<80	1280	640	320
H- 18	40960	40960	<80	160	<80	<80	1280	640	320
H- 20	40960	40960	<80	160	<80	<80	1280	640	320
H- 24	40960	40960	<80	160	<80	<80	1280	640	320
N- 9	40960	40960	80	160	<80	<80	1280	640	320
N- 18	40960	20480	<80	160	<80	<80	1280	640	320
N- 32	40960	40960	<80	160	<80	<80	1280	320	160
N-118	40960	40960	<80	160	<80	<80	640	320	160
N-119	40960	40960	<80	160	<80	<80	1280	320	160
N-288	40960	40960	<80	160	<80	80	1280	640	320
H-969	<80	<80	5120	10240	640	2560	<20	<20	<20

Table 3. Agglutinability of S-19 (Phase I) and H-969 (Phase III)
Organisms Grown on Different Media

Growth medium	Bacterial Phase	Immune rabbit serum						Artificially infected pig serum		
		No. 3 anti-K	No. 4 anti-K	No. 1 anti-H	No. 2 anti-H	No. 5 anti-O	No. 6 anti-O	B-5 anti-K	B-5 anti-K	B-7 anti-K
Trypto-soy broth	S-19	10240	10240	1280	1280	1280	1280	160	160	80
	H-969	<80	<80	10240	20480	2560	5120	<20	<20	<20
Nutrient agar	S-19	20480	10240	320	640	160	640	160	80	80
	H-969	<80	<80	10240	10240	1280	2560	<20	<20	<20
Blood agar	S-19	40960	40960	<80	80	<80	<80	1280	640	320
	H-969	<80	<80	10240	20480	1280	2560	<20	<20	<20
Charcoal agar	S-19	40960	40960	<80	80	<80	<80	1280	640	320
	H-969	<80	<80	10240	20480	1280	5120	<20	<20	<20

Table 4. Agglutinability of S-19 (Phase I) and H-969 (Phase III)
Organisms after Different Treatments

Antigen treatment	Bacterial phase	Immune rabbit serum						Artificially infected pig serum		
		No. 3 anti-K	No. 4 anti-K	No. 1 anti-H	No. 2 anti-H	No. 5 anti-O	No. 6 anti-O	B-2 anti-K	B-5 anti-K	B-7 anti-K
None	S-19	40960	40960	<80	80	<80	<80	1280	1280	640
Formol	S-19	40960	40960	<80	80	<80	<80	640	640	320
Merthiolate	S-19	10240	10240	<80	80	<80	<80	320	160	80
60°C, 1 hr.	S-19	5120	2560	<80	<80	320	1280	160	80	40
100°C, 1 hr.	S-19	2560	640	<80	<80	2560	2560	80	40	20
120°C, 10 min.	S-19	<80	<80	<80	<80	1280	2560	<20	<20	<20
None	H-969	<80	<80	20480	40960	2560	5120	<20	<20	<20
Formol	H-969	<80	<80	10240	20480	1280	2560	<20	<20	<20
100°C, 1 hr.	H-969	<80	<80	<80	<80	1280	2560	<20	<20	<20

film covering the bottom of the tube. The agglutinin titer was expressed as the reciprocal of the highest serum dilution which showed positive reaction.

RESULTS

First, the experiments were performed to see whether each strain of *B. bronchiseptica* of pig origin could react with anti-K, H and O sera. As shown in Table 2, except for H-1 and H-969, all the strains were shown to agglutinate equally with anti-K serum. Non-agglutinating H-1 and H-969 strains were of phase III or intermediate.

Then, experiments were made to find out the most sensitive and specific agglutination procedure using several kinds of antigens prepared from phase I strain S-19 and phase III strain H-969.

A preliminary study using living organisms revealed that a moderate non-specific reaction with normal pig sera was observed at 37°C, and that this was reduced to less than 1:10 at 56°C. Then, the following reactions were always made at 56°C.

As shown in Table 3, K-agglutinability was compared with living organisms grown

Table 5. Agglutinability of Formalin-killed S-19 Organisms after Storing at 4°C

Storing	Immune rabbit serum		Naturally infected pig serum*					
	No. 3 anti-K	No. 5 anti-O	N-18	N-19	N-20	N-21	N-22	N-23
1 week	40960	<80	80	320	80	40	10	10
3 months	40960	<80	80	320	160	80	20	10
6 months	40960	<80	80	320	160	80	20	10
12 months	40960	<80	80	320	160	160	40	<10

* Ten- to eighteen- week old pigs having *B. bronchiseptica* in the nasal cavity.

on four kinds of media. Antigens from blood agar and charcoal agar reacted more strongly with anti-K serum than antigens prepared from cultures on trypto-soy broth and nutrient agar.

Next, the method for obtaining the most effective antigens using organisms grown on charcoal agar were studied. As shown in Table 4, formalin-killed S-19 organisms from charcoal agar showed a K-agglutinability comparable to that of living organisms from the same medium.

On the contrary, after heating at 60°C, 100°C or 120°C, organisms from charcoal agar were not useful as antigen because of O-agglutinability. K-agglutinability showed a remarkable loss after heating at 100°C or 120°C.

To confirm the specificity of the formalin-killed antigen prepared from charcoal agar culture, sera from *B. bronchiseptica*-infected pigs were tested for K-agglutinin after absorption with the antigen. It was found that K-agglutinin disappeared completely after a single absorption with each antigen prepared from the 15 strains showing K-agglutinability.

The next experiment tested agglutinability of the formolized antigen after preserva-

Fig. 1. Comparison of Agglutinability between Living and Formalin-killed Organisms of S-19 Strain

Fig. 2. Preparation of Formalin-Killed Organisms of *B. bronchiseptica* for Agglutination Test. **Fig. 3. Method of Agglutination Test.**

Tube No.	1	2	3	4		Control
PBS	0	0.5	0.5	0.5	0.5	0.5
*Serum	0.5	0.5	0.5	0.5	0.5	0
**Antigen	0.5	0.5	0.5	0.5	0.5 Discarded	0.5
Final serum dilution	1:10	1:20	1:40	1:80		
↓						
Shaked thoroughly						
↓						
Incubated at 56°C for 2 hrs.						
↓						
Kept at 4°C overnight						
↓						
Left at room temperature for 1 hr.						
↓						
Result reading: graded 3+, 2+, 1+ and —						
Positive; $\geq 1:10$ Negative; $< 1:10$						

* Test serum in 1:5 dilution with PBS and inactivated at 56°C for 30 min.

** The concentration of the antigen was adjusted to McFarland's nephelometer No. 5 with PBS.

tion at 4 to 5°C. The antigen was prepared from charcoal agar culture of phase I strain S-19 and was tested for agglutinability at intervals during 12 months. As shown in Table 5, the agglutinability of the antigen did not show any significant changes even after 12 months of storage.

When agglutinability was compared between living and formolized organisms of strain S-19 in reaction with sera from naturally infected pigs, agglutinin titers obtained by the formolized antigen were similar to or higher than those obtained by living organisms from the trypto-soy broth culture (Fig. 1).

According to the results presented here, a new procedure of the tube agglutination test for *B. bronchiseptica* infection in pigs is shown in Figs. 2 and 3. By this procedure,

agglutinin titers of 1 : 10 or higher should be determined as positive.

DISCUSSION

KASUGA et al.^{2,3)} reported on the relationship between morphological natures and serological structures of *B. pertussis*, *B. parapertussis* and *B. bronchiseptica*. In their report, it was clearly demonstrated that after repeated subculture, phase I organism of genus *Bordetella* having a thick capsule and full capsular antigens, changed to either an intermediate phase having only a thin capsule and partial capsular antigens, or to phase III completely lacking a capsule. Also KASUGA et al.⁴⁾ reported that phase III or intermediate phase of *B. pertussis* was frequently isolated from whooping cough patients.

The present authors also noticed that phase I organisms obtained from the nasal cavities of infected pigs changed frequently to intermediate phase or phase III organisms after one or two subcultures.

In the present studies, phase variations were tested to select a stable strain useful for agglutination test. Except for two, most strains of *B. bronchiseptica* examined were found to be useful as antigens for serological diagnosis, providing that they were serologically stable phase I organisms. It is suggested that a careful examination is necessary to select a fairly stable strain being used for serological test.

In our previous paper¹⁾, a test for detecting *B. bronchiseptica* antibody in pigs was proposed using living organisms from trypto-soy broth culture. However, such living antigen seemed to be very inconvenient for practical use. As shown in Fig. 1, formalin-killed organisms from charcoal agar culture were shown to give titers similar to or higher than those obtained by living antigen prepared from trypto-soy broth culture. Consequently, providing that stable phase I strains were selected for antigen preparation, such killed antigens seem to be more useful for practical use to detect natural infection in pigs.

SUMMARY

Agglutinability between living and formolized organisms were compared with naturally or artificially infected pig sera, in consideration of bacterial strains, culture media, and treatments for antigen preparation as well as reaction temperature, specificity and stability of antigens.

Formalin-killed phase I organisms prepared from charcoal or blood agar culture were shown to be useful for the diagnosis of *B. bronchiseptica* infection in pigs.

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豚の伝染性萎縮性鼻炎の病原学的研究

IV. ホルマリン死菌抗原による凝集反応について

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(昭和47年8月19日受付)

著者らはさきに *Bordetella bronchiseptica* (以下 B 菌) 感染豚の血清学的診断についてトリプトソイブイオン培養生菌を用いる方法 (ブイオン法) を報告した. この抗原は生菌であるため保存性がなく, 試験の都度調製する必要がある, また野外試験において生菌を抗原とする点に問題があり, さらに凝集反応の判定に際しやや明瞭を欠くことなどの問題点があった. そこで死菌抗原の作成を目的として, B 菌の抗 K, O および H 血清を用いて凝集反応用菌株, 培養条件, 抗原の処理, 特異性ならびに安定性などにつき再検討し

た. ついで自然感染豚, 実験感染豚, 非感染豚および HPCD 豚血清を用いてブイオン培養生菌抗原とホルマリン死菌抗原の被凝集能を比較した. その結果, 相変異に安定な 1 相菌株を選定し, 炭末加寒天培地あるいは 5% 馬血液加トリプトソイ寒天培地発育菌のホルマリン死菌抗原がすぐれた K 被凝集能を示した. また凝集反応はホルマリン死菌抗原を McFarland No. 5 の濃度に調整し, 被検血清と 56°C, 2 時間感作する方法が良好な成績を示した.