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PARALLEL INVESTIGATIONS OF DIFFERENT SEROLOGICAL METHODS FOR DETECTING SPECIFIC ANTIBODIES AGAINST BORDETELLA BRONCHISEPTICA IN EXPERIMENTALLY INFECTED PIGLETS

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The agglutination reaction is the method used most often for detecting specific antibodies against B.bronchiseptica. Not many literature data are available on the application of other serological methods. The aim of our investigation was to study the possibility of applying the methods of CF-Kolmer, mCF, heminhibition (HI) and agar-gel precipitation (AGP) and to compare the results obtained with the method of microagglutination (MA), which is used as the standard method for detecting specific antibodies against B. bronchiseptica.

Blood serum was obtained from experimentally infected piglets as well as from piglets in co-habitation. Blood samples were taken at fifteen-day-intervals until day 165 of the infection. The appropriate antigens were prepared from the virulent strain V of B. bronchiseptica, which was also used for experimental infection.

Using the MA method, positive results were obtained for 97,3% of the investigated sera. The agglutinin level increased after infection and reached maximal values on day 45 (GMT=163,14), after which it decreased gradually and on day 165 it was GMT=22,63. Using the mCF method, antibodies against B. bronchiseptica were found in 81,1% of the investigated piglets as well as higher titers compared to the standard CF-Colmer method. Using the HI method, positive results were obtained for 69,4% of the piglets. The values of antibody titers ranged from 1:4 to 1:64. The AGP method, as applied, proved to be insufficiently sensitive. Positive results were obtained for 30.6% of the investigated animals.

Key words: B. bronchiseptica, piglets, MA, CF-Colmer, mCF, HI, AGP

INTRODUCTION

Since 1956. B. bronchiseptica has been considered to be the primary causative agent of atrophic rhinitis (Switzer et al., 1956.). This was later confirmed also by other researchers, (Brassine et al., 1976, Kemeny, 1972. and Mihajlović

et al., 1978.). B. bronchiseptica is widely spread in pig herds and is localized on the epithelial surface of the respiratory tract. Atrophic rhinitis is a very significant health and economic problem that has been registered almost all over the world. Making a diagnosis for atrophic rhinitis is mainly based on a data combination obtained by clinical and laboratory investigations. The clinical picture gives very important data, taking into consideration that signs of the disease are noticeable and characteristic, but clinical changes are not always, and need not be, present in atrophic rhinitis. For the etiological diagnostics of atrophic rhinitis the isolation of B. bronchiseptica is very important. Many researchers have pointed to difficulties in the isolation of the causative agent. Opinions on the value of the serodiagnostics of atrophic rhinitis are not uniform. The agglutination reaction is the most often used method for detecting specific antibodies against B. bronchiseptica (Brandenburg, 1978; Jenkins, 1977; Kang et al., 1970; Kemeny, 1973).

Not many data are available on the application of other serological methods. Bercovich and Oosterwond (1977), applied the CF-method for detecting antibodies in sera of vaccinated pigs. The fact that in the available literature there are no data about the use of other serological methods for detecting specific antibodies against B. bronchiseptica was the reason to lead our investigations in that direction.

The aim of our investigations was to study the possibillity of applying the methods of CF-Colmer, mCF, HI and AGP for detecting specific antibodies against B. bronchiseptica.

MATERIAL AND METHODS

Experimental animals. In our investigations 30 piglets were used, from which 22 were inoculated and 8 lived in co-habitation. The two and three-day-old piglets were inoculated intranasally with 0,5 ml of the virulent strain V of B. bronchiseptica in each nostril in; the phase of inhalation. Blood samples were taken on the day of farrowing and afterwards at fifteen-day intervals until day 165 of the infection.

Methods

1. The method of microagglutination which is most often used in serological diagnostics of atrophic rhinitis, was taken as the standard procedure. (Brandenburg 1978, Lind 1977, Vidić et. al., 1994).

2. Heminhibition (HI)

Serum: To 1 ml of the sera that had previously been inactivated, 0,1 ml of 10% suspension of ram erithrocytes was added. This mixture was incubated for 1 hour at 4^0C and afterwards centrifuged and the supernatant used in the reaction.

Antigen: To prepare the antigen, B. bronchiseptica in phase I, after cultivation on blood agar with 5% of bovine blood for 24 h, was used. The colonies grown were rinsed out with 3 ml of physiological saline solution and a thick suspension of bacteria (10^{10} of bacteria / ml) was used in the reaction of hemagglutination. The hemagglutination test was performed in test tubes where double dilutions of

bacteria with physiological saline solution were prepared (1:4 do 1:2048) and to each test-tube 0,4 ml of a 0,5% suspension of ram erithrocytes was added. The reaction was read after 1 hour incubation at room temperature. The reaction included control erithrocytes. The highest dilution of bacteria, where total hemagglutination appeared, was marked as one hemagglutination unit. To carry out the heminhibition procedure, a dilution of bacterial suspension which contained four hemagglutination units was used.

Method: Double dilutions of prepared sera were made in test-tubes (0,2 ml of physiological saline + 0,2 ml of the serum, from which 0,2 ml was transferred to the next test-tube containing 0,2 ml of physiological saline serially up to the last test-tube). Antigen (4 HA_j) in 0,2 ml was added to the serum dilutions and they were then incubated for 30 min. at room temperature. Afterwards, 0,4 ml of 0,5% suspension of ram erithrocytes was added to serum dilutions which were then incubated at room temperature for 1 h when the results were read. Controls for the serum, the antigen and erithrocytes as well as positive and negative sera were included.

3. CF-method

a) The method CF-Colmer was applied. The culture of B. bronchiseptica multiplied in tryptose phosphat agar was used as the antigen. After 48 hours the colonies were rinsed out with 3-4 ml of sterile distilled water and a suspension of density Mc. F.5 was prepared. The suspension was homogenized in a magnetic mixer and placed in the Koch's pot for 2 hours. When it was cooled down to about 50^0C, 1 g NaCl and 5 ml of 5% phenol was added to 100 ml of suspension. This suspension was centrifuged for 30 min. at 3000 rpm and the supernatant was used as antigen. The antigen was titrated and 2 units was used. The complement and the hemolusine were also adjusted to contain 2 units in the working volume (0,2 ml). Controls were included in the work (positive and negative sera, serum and antigen controls). The finding of specific antibodies in a serum dilution 1:8 and greater was considered a positive result.

b) An mCF method modified according to Nicol et al., (1971) was used. In this procedure the same antigen as in the CF-Colmer method was applies. The test was carried out using microplates with U-shaped bottoms. The investigate sera were inactivated for 30 minutes at 58^0C and then dilutions with physiological saline from 1:2 to 1:256 were prepared. Fresh guinea pig sera were used as complement that was titrated before the work, adjusted to contain 2 units in the working volume to which 5% of calf serum was then added. A suspension of equal parts of 2,5% ram erithrocytes and the hemolysin that contained 4 units in the used volume was applied as a hemolytic system.

An equal quantity (0,005 ml) of the antigen and the complement was added to the prepared serum dilution (0.025 ml). The plates were incubated for 18-20 h at 4^0C. The mixture of equal parts of hemolysin and 2,5% ram erithrocytes (0,05 ml) was placed in each well, the plate was shaken and incubated for 30-45 min. at 37^0C and then the result was read. Controls were included. The finding of specific antibodies in serum dilutions 1:8 and greater was considered a positive result.

4. Agar-gel precipitation

A medium of 1% Noble agar (Difco) in phosphate-buffered saline (PBS) solution (pH 7,2) was used. The medium was sterilized for 30 min. at 100^0 C and 16 ml was poured into petri dishes. Seven holes with a diameter of 7 mm were made. The six peripheral holes were at a distance of 5 mm from the central hole. The antigen (50 μl) was placed in the central hole and the control positive serum (50 μl) as well as the test sera (50 μl) in the peripheral holes. Petri dishes were left at room temperature and the results were read after 48 and 72 hours.

Antiserum (the control positive serum): A hyperimmune gilt serum was used in the reaction. The antigen for immunization was prepared from a 48 hour old culture of B. bronchiseptica from blood agar. The grown colonies were rinsed out with physiological saline that contained 0,3% of formalin, and for immunization a suspension of bacteria of density Mc. F. 5 was used. The gilts were immunized with an inoculum that consisted of equal parts of the bacterial suspension and complete Frenud's adjuvant, subcutaneously in the quantity of 2 ml. 6 times at 7-day-intervals. On the 7th day after the last application blood samples were collected and the blood serum was separated. The amount of 2 ml was stored at -20^{0}C.

Antigen: A 48 hour old culture of B. bronchiseptica from blood agar was rinsed out with 3 ml of physiologic saline that contained 0,3% of formalin. This thick bacterial suspension was exposed to ultrasound (3 times for 2 minutes each, at maximal amplitude) and then centrifuged at 12,000 rpm for 30 min. The clear supernatant was held at 100^{0}C for 1 hour and used as the antigen in the precipitation reaction.

RESULTS AND DISCUSSION

The results showed that agglutinins, complement binding antibodies, antibodies that present hemagglutination and precipitins can be detected in blood sera from infected piglets and piglets in co-habitation by use of different serological methods.

The applied methods and appropriate antigens were checked with hyperimmune gilt and rabbit sera prior to this investigation (Table 1).

Using the MA method, on day 15 of the infection agglutinins were detected in infected piglets as well as in piglets in co-habitation (Table 2). These results confirm the investigations of Smith et al., (1972.) and Tornoe et al., (1976), who found that the infectious agent spread quickly from experimentally infected piglets to other animals in the litter. These piglets showed symptoms of the disease on day 9 of the infection, whereas Giles at al., (1980) found already after 3 days a spontaneous infection in piglets that were in contact with animals from the same or a neighboring litter. The highest agglutinin levels were registered on days 45 and 60 of infection, after which the level decreased. Nevertheless, it was possible to detect it on day 165 of infection. Martineau et al., (1982) and Kemeny (1973). obtained similar results in their investigation. In natural infection, antibodies are discovered later (Kang et al., 1966; Shimizu et al., 1971), which is attributed to inadequate stimulation in the early phase of the infection, taking into consideration that B. bronchiseptica is localized on the surface of the epithelium.

The observed differences could be a consequence of the route of infection, because in experimental infection there is a high dose of inoculum. On the contrary, data on weak immunological reactivity after experimental infection were presented by Popović et al., (1981) and Shimizu et al., (1971).

Table. 1. Titres of agglutinin, CF - antibodies, hemagglutinin and precipitinin in blood sera of piglets and rabbits immunized with strain V of B. bronchiseptica

		METHODS				
		MA	CF	m CF	HI	AGPT
Hyperimmune serum of gilts	1 G	1024	32	64	32	+
	2 G	512	16	16	32	+
	3 G	2048	128	256	64	+
	4 G	2048	64	256	64	+
	5 G	4096	256	512	128	+
	6 G	8192	512	2048	128	+
	G M T	2048.00	90.51	203.66	64.00	
Hyperimmune serum of rabbits	1 R	1024	128	128	32	+
	2 R	4096	512	1024	64	+
	3 R	1024	32	128	8	+
	4 R	2048	128	256	64	+
	G M T	1722.15	128	256.00	32.00	

MA = Microagglutination
AGPT = Agar gel precipitation test
mCF = micro CF
CF = Colmer

Table 2. Agglutinin results for blood sera of artificially infected piglets and piglets in co-habitation by use of the MA method.

	Infected piglets			Pigl. in co-habitation			Total		
Day p/i	No. of Animals	Titre from to	GMT	No. of Animals	Titre from to	GMT	No. of Animals	Positive No.	Positive %	GMT
15	18	16 – 64	25.46	8	4 – 32	13.45	26	23	88.5	20.83
30	15	64 – 256	97.00	7	64 – 128	86.22	22	22	100	93.05
45	14	64 – 256	172.44	6	64 – 256	144.01	20	20	100	163.14
60	10	64 – 512	137.19	5	64 – 128	111.43	15	15	100	128.00
75	9	32 – 128	80.45	5	32 – 128	73.52	14	14	100	77.71
90	9	32 – 128	59.30	4	64	64.00	13	13	100	66.55
105	6	16 – 64	45.25	3	32 – 64	50.91	9	9	100	46.85
120	6	16 – 64	36.00	3	32 – 64	40.22	9	9	100	37.27
135	5	16 – 32	27.86	2	32	32.00	7	7	100	29.04
150	4	16 – 32	26.91	2	16 – 32	22.63	6	6	100	25.46
165	4	16 – 32	22.63	2	16 – 32	22.63	6	6	100	22.63

In tables 3 and 4, the results using the CF-Colmer method and the modified micro method (mCF) are presented.

It can be noticed that the mCF method gives a higher percentage of positive animals and higher titers of specific antibodies were determined. The differences obtained could be explained from the fact that we added to the mCF complement fresh calf serum that has the characteristic to prevent the procomplementary and

anticomplementary activity of swine serum, as reported by Boulanger et al., (1968.) and Nicolet et al., (1971).

Table 3. Antibody levels in blood sera of artificially infected piglets and piglets in co-habitation found by use of the CF method (Colmer)

Day p/i	Infected piglets			Pigl. in co-habitation			Total			
	No. of Animals	Titre from to	GMT	No. of Animals	Titre from to	GMT	No. of Animals	Positive No.	Positive %	GMT
15	18	< 4 – 16	3.84	8	< 4 – 4	1.68	26	8	30.8	2.99
30	15	4 – 32	13.93	7	8 – 16	10.76	22	20	90.9	12.82
45	14	8 – 64	29.04	6	16 – 32	18.00	20	20	100	25.11
60	10	8 – 32	21.11	5	16 – 32	21.11	15	15	100	21.11
75	9	8 – 16	12.73	5	8 – 16	12.12	14	14	100	12.47
90	9	4 – 16	8.63	4	8 – 16	9.51	13	12	85.7	8.88
105	6	< 4 – 8	3.55	3	< 4 – 8	3.18	9	4	44.4	3.43
120	6	< 4 – 8	1.78	3	< 4 – 8	2.00	9	2	22.2	1.85
135	5	< 4 – 8	3.03	2	8	8.00	7	4	57.1	4.00
150	4	< 4 – 8	2.38	2	< 4 – 8	2.00	6	1	16.6	2.25
165	4	< 4 – 8	1.64	2	< 4	0.0	6	1	16.6	1.41

Table 4. Antibody levels in blood sera of artificially infected piglets and piglets in co-habitiation found by use of the mCF method

Day p/i	Infected piglets			Pigl. in co-habitation			Total			
	No. of Animals	Titre from to	GMT	No. of Animals	Titre from to	GMT	No. of Animals	Positive No.	Positive %	GMT
15	18	< 4 – 16	5.24	8	< 4 – 8	2.18	26	12	46.1	4.00
30	15	8 – 64	23.10	7	16 – 32	4.57	22	22	100	23.26
45	14	16 – 128	60.97	6	32 – 64	56.88	20	20	100	59.71
60	10	32 – 128	59.71	5	32 – 64	55.71	15	15	100	58.48
75	9	16 – 64	36.76	5	16 – 32	24.25	14	14	100	32.00
90	9	16 – 32	25.46	4	16 – 32	19.03	13	13	100	23.26
105	6	16	16.00	3	16	16.00	9	9	100	16.00
120	6	8 – 16	12.73	3	8 – 16	12.73	9	9	100	12.73
135	5	< 4 – 16	6.96	2	8 – 16	11.31	7	6	85.7	8.00
150	4	4 – 8	6.72	2	8	8.00	6	5	83.3	7.12
165	4	4 – 4	5.66	2	4 – 8	5.66	6	3	50.0	5.66

Table 5. Antibody levels in the blood sera of artificially infected piglets and piglets in co-habitation found by use of the HI method

Day p/i	Infected piglets			Pigl. in co-habitation			Total			
	No. of Animals	Titre from to	GMT	No. of Animals	Titre from to	GMT	No. of Animals	Positive No.	Positive %	GMT
15	18	< 4 – 8	2.33	8	< 4 – 4	2.38	26	4	15.4	2.34
30	15	4 – 32	10.56	7	8 – 32	17.63	22	18	81.8	12.47
45	14	16 – 64	33.60	6	16 – 64	28.44	20	20	100	32.00
60	10	16 – 32	29.86	5	16 – 32	21.11	15	15	100	26.54
75	9	8 – 32	11.71	5	8 – 32	16.00	14	14	100	14.52
90	9	< 4 – 16	8.68	4	8 – 16	13.45	13	11	84.6	9.92
105	6	< 4 – 8	5.66	3	8	8.00	9	8	88.9	6.36
120	6	< 4 – 8	5.03	3	8	8.00	9	7	77.8	5.86
135	5	< 4 – 8	4.60	2	4 – 8	5.66	7	4	57.1	4.86
150	4	< 4 – 8	3.36	2	< 4 – 4	2.00	6	1	16.7	2.83
165	4	< 4 – 4	1.41	2	< 4 – 4	2.00	6	0	0.0	1.59

Table 6. Finding of precipitin in blood sera of infected pigs in co-habitation by the AGP method

	Day after infection											
	15	30	45	60	75	90	105	120	135	150	165	Total
No. of pigs	26	22	20	15	14	13	9	9	7	6	6	**147**
Positive												
No.	0	8	16	11	5	3	2	0	0	0	0	**45**
%	0	36.4	80.0	73.3	35.7	23.1	22.2	0	0	0	0	**30.6**

The results obtained by the HI method are presented in table 5. It is obvious that the infection with B. bronchiseptica can be demonstrated using this method. Antibodies were detected on day 15 of the infection in 4 animals with the titer 1:8 that was marked as positive, whereas the highest titer mean value determined on day 45 of the infection (GMT=32). Afterwards, the antibody level decreased but persisted up to day 150 of the infection. Taking into consideration that we found no literature data about the use of this method, on the basis of our investigations we are of the opinion that further investigations should be directed towards obtaining a purified antigen that that would contain a capsule, i. e. fimbriae. This would certainly influence the sensitivity and specificity of the reaction.

The finding of precipitin in blood sera of experimental animals is shown in table 6. Using this method antibodies were found on day 30 of the infection in 8 out of 22 investigated sera, whereas on day 150 antibodies were detected only in two animals. It should be mentioned that precipitation lines were clear and visible. However, on the basis of the obtained results we consider that this method is insufficiently sensitive.

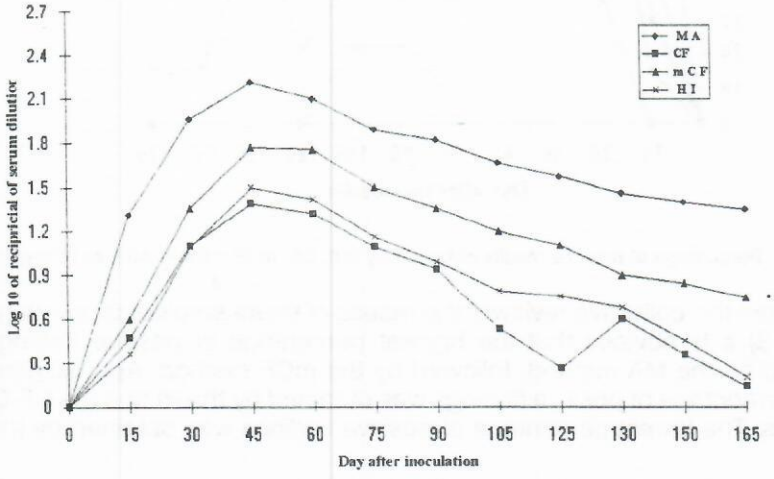

Figure 1. Average value of antibody titres in the blood of piglets obtained by MA, CF, mCF and HI methods

In Figure 1. the degree and the duration of the immunological response in experimental piglets using four serological methods are shown. It is evident that

the antibody level increases after infection, reaches its maximal values on day 45 and, with a tendency to decrease stays evident until day 165 using the MA, the mCF and the CF-Colmer methods Using the HI method antibodies could be detected until day 150 and using the AGP method until day 105 of the infection. The highest titers were found using the MA method and the lowest using the CF-Colmer method.

In our investigations, detection of agglutinin in serum dilutions 1:20 and higher, and in HI-reactions, CF-Colmer and mCF the titer 1:8 and higher were considered positive titers. Using these criteria in the estimation of serological reactions, positive findings were obtained on day 165 of the infection by using microagglutination (Figure 2). This is very important, because the infection can be found in a period when the isolation of B. bronchiseptica from nasal swabs is not successful. Kang et al., (1961) and Kemeny, (1973) also obtained similar results in their investigations. These authors found a correlation between antibody findings and pathological changes on swine conchae of 77,3%.

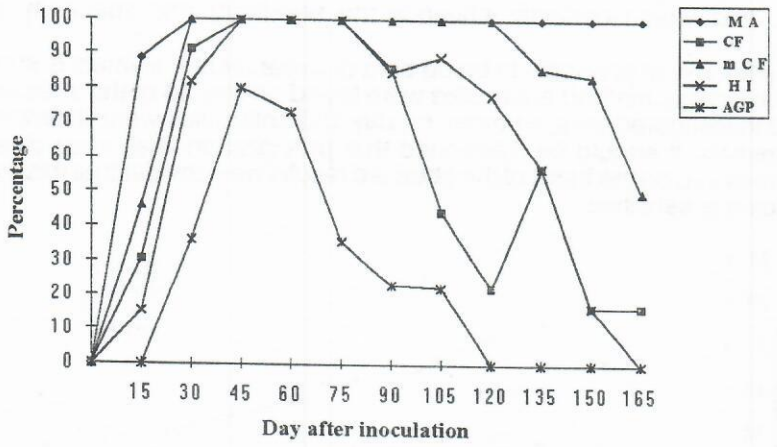

Figure 2. Percentage of positive results obtained by MA, CF, mCF, HI and AGP at 15-day i;ntervals

From the collective reviewof the results of these serological investigations (Figure 3) it is obvious that the highest percentage of positive findings was obtained by the MA method, followed by the mCF method. Approximately the same percentage of positive findings was obtained by the HI and the CF-Colmer methods. The lowest percentage of positive findings was obtained by the AGP method.

It is not hard to conclude from the papers of numerous researchers that the validity of serological investigations can be used for determination of the spread of infection with B. bronchiseptica. Our results justify the use of agglutination in serodiagnostics of atrophic rhinitis, which was also confirmed by the results of pathomorphological investigations (Vidić et al., 1966). However, just detecting

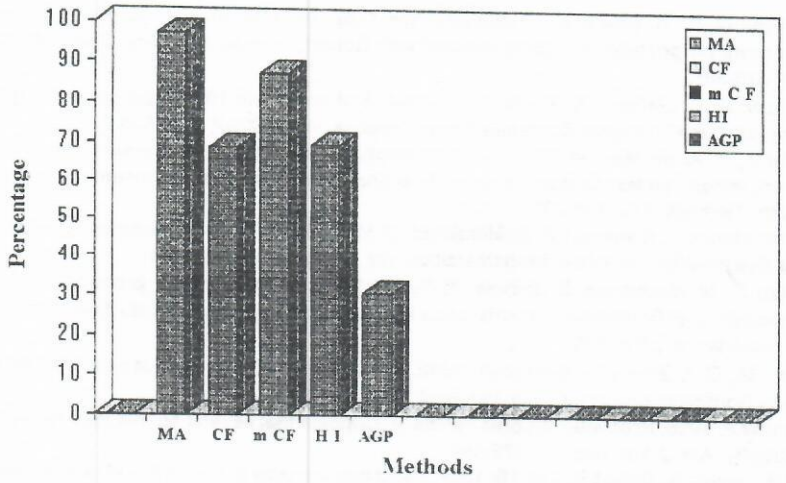

Figure 3. Percentage of positive results obtained by MA, CF, mCF, HI and AGP for the whole investigation period

specific antibodies in the serum of infected animals is an insufficient parameter for making a conclusion about the clinical condition of an individual or a herd. Strain, virulence, age of infected animals, housing conditions and other non-specific factors are significant for the appearance and the intensity of the infection and because of this the evaluation of serological findings should be performed in parallel with bacteriological and clinical investigations.

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UPOREDNO ISPITIVANJE RAZLIČITIH SEROLOŠKIH METODA ZA DOKAZIVANJE SPECIFIČNIH ANTITELA PROTIV B. BRONCHISEPTICA U VEŠTAČKI INFICIRANE PRASADI

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SADRŽAJ

Ispitivana je mogućnost primene Reakcije vezivanja komplementa (RVK) po Colmer-u, mikro RVK (mRVK), heminhibicije (HI) i agar-gel precipitacije (AGP) u cilju dokazivanja specifičnih antitela protiv B. Bronchiseptica u krvnom serumu veštački inficirane prasadi. Dobijeni rezultati upoređivani su sa rezultatima dobijenim metodom mikroaglutinacije (MA), kao standardnom metodom za ispitivanje specifičnih antitela protiv B. bronchiseptica. Metodom mikroaglutinacije za ukupan period ispitivanja ustanovljeno je 97,3% pozitivnih seruma. Dobijene vrednosti ukazuju da nivo aglutinina raste nakon zaražavanja, dostiže maksimalne vrednosti 45. dana (Srednja geometrijska vrednost-SGV = 163,14), zatim postepeno opad ai 165. dana iznosi SGV = 22,63. Primenom mRVK ustanovljena su antitela protiv B. bronchiseptica kod 81,1% ispitivane prasadi kao i viši titri u odnosu na standardni metod RVK po Colmer-u. Metodom heminhibicije pozitivne vrednosti dobijene su kod 69,4% životinja. Vrednost titra antitela kretala se od 1:4 do 1:64. Metod agar-gel precipitacije (AGP) pokazao se nedovoljno osetljiv iz razloga što su specifična antitela protiv B. bronchiseptica ustanovljena samo kod 30,6% pregledanih životinja.