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COMPARISON OF DIFFERENT MEDIA AND ENVIRONMENTAL CULTURAL CONDITION ON THE YIELD OF *HAEMOPHILUS PARAGALLINARUM* STRAIN

S.M SHAFAY[1], ELHAM A.EI EBIARY[1] AND S.M. GERGIS[2]

1 Central Laboratory for control of Veterinary Biologics, Agricultural Reasrch Centre, Giza Egypt.

2 Veterinary Serum and Vaccine Research Institute, Agricultural Reasrch Centre, Giza, Egypt.

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Abstract

This study revealed that the best cultural conditions to produce a high yield of *H.paragallinarum* (HP) bacterial cells for bacterin or antigen preparation was the fermentative method. The best yield on dry weight basis was obtained by chicken meat infusion broth "CMI" with 0.5% chicken serum and brain heart infusion broth "BHI" with 1% horse serum after spreger 10% CO_2 gassing in the media for four hours and incubation at 37°C for 18 hours. Cultures produced by this method and diluted twice produce an effective protective bacterin and sensitive specific antigen. This finding has an economical value.

INTRODUCTION

Haemophilus paragallinarum is a fastidious organism which is usually grown using complex media containing serum or other fresh blood products (Rimler *et al.*, 1975). It grows on tryptose agar or brain infusion broth without serum or feeder cultures provided that NADH is incorporated into the medium (Page *et al.*, 1963). It appears from previous studies that there is a great controversy on the importance of CO_2 for growth of *H.paragallinarum* in both media (Rimler *et al.*, 1976). Chicken meat infusion broth supplemented with chicken serum was utilized by Matsumoto and Yamamoto (1975) to propagate the organism used in bacterin preparations.

The purpose of the present study was to develop a fermentor method for increasing the growth of *H.paragallinarum* to be used either for vaccine or antigen preparation and to compare the preparations produced by this method with those of static method (Richard *et al.* 1976).

MATERIALS AND METHODS

Bacterial strains

H.paragallinarum strains "W" (Serovar "A") and "Modesto" (serovar "C")

(Kume *et al.*, 1980 and Sawata *et al.*, 1980) were kindly obtained from Intervet International B.V., Boxmeer, Holland.

Culture media

Four types of media were used for antigen and bacterin preparations. Chicken meat infusion broth was prepared as described by Matsumoto and Yamamoto (1975) and Ortiz and Yamamoto (1974). Brain heart infusion broth was supplied by Difco, Scotland, Batch No. 10E 7393B. Tryptose phosphate broth was obtained from Difco, Michigan, USA, Batch No. 0061-01. Thioglycolate broth medium was purchased from Oxoid, England, Batch No. CM173.

Preparation of seed material

Lyophilized strains were rehydrated with 1 ml of tryptose phosphate broth/ ampoule. Then, the rehydrated strains were inoculated in three liters of four types of media (CMI, BHI, TPB and TBM), one liter for each condition. Each litre contained nicotinamide adenine dinucleotide reduced form (NADH) or V-factor (Sigma, USA, Batch No. 63178) 2.5 ug/ml and 0.5% in chicken serum or 1% horse serum (X-factor).

Methods of cultivation

The inoculated media were incubated at three different conditions. The first method, referred to as static method, was done by cultivating the organism in still culture and incubated in CO_2 incubator for 24 hours at 37°C (Rimler, 1982). The second method referred to as fermentative method, was done by a gas consisting of 10% CO_2 was sparger aereated through the medium for 2 hours during incubation at 37°C. The flask was closed upon cessation of gassing. Then, the flask was further incubated for 18 hours. The third method was done by aereation CO_2 gas through the medium for 4 hours during incubation. The bacterial cells concentration in one ml of culture equalised the density of MacFarland tube No. 3 when culture was incubated in Co_2 incubator, while, it equalised No. 4 when Co_2 was aereated in media for 4 hours. Following incubation, each culture was centrifuged at 6, 000 r.p.m. for 15 minutes and the dry weights of *H.paragallinarum* bacterial cells were measured. Comparison was done between different conditions to determine the optimum one that gave heavy yield of growth of *H.paragallinarum* bacterial cells.

Detection of bacterial growth

After growth was completed, different culture media were concentrated by centrifugation at 6000 xg for 10 minutes at 4°C and washed 3 times on 0.02 M PBS. The pellet was then dried by continuous air flow at 40°C. Then, the dried pellets were weighed.

Vaccine preparation

The method of Matsumoto and Yamamoto (1975) and Rimler *et al.* (1975) was adopted. Briefly, bacterial cultures were inactivated by adding formalin to a final concentration of 0.25%. Freund's incomplete adjuvant at a concentration of 50% of the final volume was emulsified with cultures. The final concentration of *Haemophilus paragallinarum* strains in vaccine was adjusted to 3.96×10^8 CFU/dose of "W" and 3.93×10^8 CFU/dose of "Modesto" strain (Richard *et al.*, 1976).

Antigen preparation

The technique described by Iritani *et al.* (1977) was followed using bacterial cells grown by fermentative method.

Experimental chicken

Sixteen White Leghorn, 6-8 weeks of age were used in this experiment. They neither have a history of infection with *H.paragallinarum* nor of vaccination.

RESULTS AND DISCUSSION

Bacterins which can protect the upper respiratory tract of chicken against challenge with virulent *H.paragallinarum* have been developed (Matsomoto and Yamamoto, 1975). Chicken meat infusion broth supplemented with chicken serum was utilized by them to propagate organism used in bacterin preparation. The bacterin containing at least 10^8 CFU of *H.paragallinarum* inactivated by thimersal or formalin and potentiated by aluminium hydroxide or incomplete Freund's adjuvant was found to provide protection. The most important problem encountered in vaccine preparation was to obtain heavy growth of *H.paragallinarum* organism. In present study, a method for increasing growth of the organism was adopted by applying a fermentative technique for propagation of organism in stead of the static culture method (Richard *et al.*, 1976).

From results tabulated in Tables 1, 2 and 3, the dry weight (g) of bacterial cells per liter of each medium grown was obtained by either static or fermentative method with addition of either of chicken or horse serum as well as without serum. It can be concluded that the best yield of bacterial cells obtained when culture on CMI with 0.5% chicken serum 0.65 g/L used, while, 0.61 with 1% horse serum and 0.60 without serum . This yield was obtained by static method, while by fermentative method for 2 hours 0.81, 0.78 and 0.77, respectively, but high yield was obtained when fermentative for 4 hours 0.83, 0.79 and 0.78, respectively.

Also, high yield was obtained when culture on BHI by static method 0.59, 0.63 and 0.58, but by fermentative method 2 hours 0.77, 0.82 and 0.74 and, when fermentative for 4 hours 0.79, 0.85 and 0.76, respectively. Least yield was obtained when used TPB 0.41, 0.49 and 0.45 by static method and 0.66, 0.69 and 0.62 when used fermentative method 2 hours, but when used for 4 hours 0.70, 0.70 and 0.65, respectively. Least yield was obtained when used TPB 0.41, 0.49 and 0.45 by static method and 0.66, 0.69 and 0.62 when used fermentative method 2 hours, but when used for 4 hours 0.70, 0.70 and 0.65, respectively. In case of TBM gave 0.40, 0.42 and 0.38 yield cells/g/L) in static method, but fermentative method for 2 hours gave 0.61, 0.51 and 0.49 while when fermentative for 4 hours gave yield of cells 0.62, 0.55 and 0.53, respectively. Finally, the best yield in case of CMI media with 0.5% chicken serum by fermentative method 4 hours, followed by BHI with 1% horse serum by fermentative method 4 hours, but least yield in case of TPB and TBM media by static and fermentative method. Also, no significant difference was noticed in cell yield when added chicken or horse serum and without addition.

It can be concluded from these results that *H.paragallinarum* organism grows better by fermentative method in an increased CO_8 atmosphere as compared with still culture. When grown under CO_2 tension in broth media lacking serum by fermentative method fewer organisms were required to initiate growth and the best growth on dry weight basis could be obtained. This finding suggests the possibility of substituting addition of serum by using fermentative culture method. Results of the present study agree with previous observation of Rimler *et al.* (1976). The growth yield obtained by the fermentative method gave twice the cell numbers of organism grown by static method. This yield was more than 10^8 CFU required for an effective bacterin. Cell numbers of the culture produced by this method was diluted twice for use in vaccine preparation. Data illustrated in Tables 4-7 revealed the immunizing efficacy of the diluted culture produced by the fermentative method which was approximately similar to the geometric mean antibody titres in sera of chicken vaccinated with vaccine prepared by the static method. From economic point of view, cultures produce by the fermentative method can prove, double the amount of vaccine produced by the static method.

Table 1. Dry weight of *H.paragallinarum* culture ("W" and "Modesto") strains in different types of media incubated in Co_2 incubator.

Types of media	Dry weight (g) of cells yield per liter		
	Chicken serum (X-factor) 0.5 %	Horse serum (X-factor) 1 %	Without serum (X-factor)
CMI	0.65	0.61	0.60
BHI	0.59	0.63	0.58
TPB	0.41	0.49	0.45
TBM	0.40	0.42	0.38

Table 2. Dry weight of *H.paragallinarum* culture ("W" and "Modesto") strains in different types of media fermentative for 2 hours.

Types of media	Dry weight (g) of cells yield per liter		
	Chicken serum (X-factor) 0.5 %	Horse serum (X-factor) 1 %	Without serum (X-factor)
CMI	0.81	0.78	0.77
BHI	0.77	0.82	0.74
TPB	0.66	0.69	0.62
TBM	0.61	0.51	0.49

Table 3. Dry weight of *H.paragallinarum* culture ("W" and "Modesto") strains in different types of media fermentative for 4 hours.

Types of media	Dry weight (g) of cells yield per liter		
	Chicken serum (X-factor) 0.5 %	Horse serum (X-factor) 1 %	Without serum (X-factor)
CMI	0.83	0.79	0.78
BHI	0.79	0.85	0.76
TPB	0.70	0.70	0.65
TBM	0.62	0.55	0.53

GMI : Chicken Meat Infusion Broth. BHI: Brain Heart Infusion Broth.

TPB : Tryptose Phosphate Broth. TBM: Thioglycolate Broth Medium.

Table 4. Results of tube agglutination test in chicken vaccinated with coryza vaccine prepared by fermentative method by using "w" antigen.

Weeks post-vaccination	Number of tested serum	Serum samples showing agglutination antibodies at each titer									Geometric mean antibody titers
		0	20	40	80	160	320	640	1280	2560	
Pre-vaccination	30	30	-	-	-	-	-	-	-	-	-
1ry vacc.	20	-	-	-	-	4	8	7	1	-	379.1
2nd week	10	10	-	-	-	-	-	-	-	-	0.0
Control											
4 th week	20	-	-	-	1	1	10	7	1	-	398.0
Control	10	10	-	-	-	-	-	-	-	-	0.0
2 ry vacc.											
2nd week	20	-	-	-	-	3	8	7	1	1	430.0
Control	10	10	-	-	-	-	-	-	-	-	0.0
4 th week	20	-	-	-	-	2	9	7	1	1	442.0
Control	10	10	-	-	-	-	-	-	-	-	0.0

Table 5. Results of tube agglutination test in chicken vaccinated with coryza vaccine prepared by fermentative method by using "Modesto" antigen.

Weeks post-vaccination	Number of tested serum	Serum samples showing agglutination antibodies at each titer									Geometric mean antibody titers
		0	20	40	80	160	320	640	1280	2560	
Pre-vaccination	30	30	-	-	-	-	-	-	-	-	-
1ry vacc.	20	-	-	-	-	5	10	2	3	-	355.0
2nd week	10	10	-	-	-	-	-	-	-	-	0.0
Control											
4 th week	20	-	-	-	-	7	6	3	4	-	379.0
Control	10	10	-	-	-	-	-	-	-	-	0.0
2 ry vacc.											
2nd week	20	-	-	-	-	4	7	7	1	1	428.0
Control	10	10	-	-	-	-	-	-	-	-	0.0
4 th week	20	-	-	-	-	4	6	7	2	1	451.0
Control	10	10	-	-	-	-	-	-	-	-	0.0

Table 6. Results of tube agglutination test in chicken vaccinated with coryza vaccine prepared by fermentative method by using "W" antigen.

Weeks post-vaccination	Number of tested serum	Serum samples showing agglutination antibodies at each titer									Geometric mean antibody titers
		0	20	40	80	160	320	640	1280	2560	
Pre-vaccination	30	30	-	-	-	-	-	-	-	-	-
1ry vacc.	20	-	-	-	-	5	8	7	-	-	343
2nd week	10	10	-	-	-	-	-	-	-	-	0.0
Control											
4 th week	20	-	-	-	1	2	10	7	-	-	368
Control	10	10	-	-	-	-	-	-	-	-	0.0
2 ry vacc.											
2nd week	20	-	-	-	-	2	10	8	-	-	394
Control	10	10	-	-	-	-	-	-	-	-	0.0
4 th week	20	-	-	-	-	1	10	9	-	-	422
Control	10	10	-	-	-	-	-	-	-	-	0.0

Table 5. Results of tube agglutination test in chicken vaccinated with coryza vaccine prepared by fermentative method by using "Modesto" antigen.

Weeks post-vaccination	Number of tested serum	Serum samples showing agglutination antibodies at each titer									Geometric mean antibody titers
		0	20	40	80	160	320	640	1280	2560	
Pre-vaccination	30	30	-	-	-	-	-	-	-	-	-
1ry vacc.	20	-	-	-	-	6	11	1	2	-	320
2nd week	10	10	-	-	-	-	-	-	-	-	0.0
Control											
4 th week	20	-	-	-	-	7	8	3	2	-	343
Control	10	10	-	-	-	-	-	-	-	-	0.0
2 ry vacc.											
2nd week	20	-	-	-	-	4	8	7	1	-	368
Control	10	10	-	-	-	-	-	-	-	-	0.0
4 th week	20	-	-	-	-	4	7	7	2	-	394
Control	10	10	-	-	-	-	-	-	-	-	0.0

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مقارنة بين البيئات المختلفة وظروف الزرع علي نمو ميكروب الهيموفيليس بارا جالينيرم

سامي محمد شافعي[1]، ساهر مكين جرجس[2]، إلهام عطا الإبياري[1]

١ المعمل المركزي للرقابة علي المستحضرات الحيوية البيطرية-مركز البحوث الزراعية-جيزة - مصر.

٢ معهد بحوث الأمصال واللقاحات البيطرية - مركز البحوث الزراعية - جيزة - مصر.

أثبتت الدراسة أن طريقة التخمر هي أفضل الطرق التي يجب إتباعها في تحضير أعلي كثافة خلوية من ميكروب الهيموفيليس بارا جالينيرم المستخدم في إنتاج البكتريين والأنتيجينات وكانت أحسن كثافة خلوية تم الحصول عليها بإستخدام وسط غذائي شربة منقوع لحم الدجاج (مضاف إليها ٠,٥ ٪ مصل دجاج) وشوربة منقوع المخ والقلب (مضاف إليه ١٪ مصل خيول) وضخ ١٠٪ غاز ثاني أكسيد الكربون في الوسط الغذائي لمدة ٤ ساعات ثم إستمرار التحضين عند ٣٧° م لمدة ١٨ ساعة دون ضخ الغاز.

وبإتباع طريقة التخمر يتم تخفيف الخلايا مرتين وبذلك يتم مضاعفة الأنتاج للبكتريين ويكون ذا قوة مناعية فعاله وكذلك إنتاج أنتيجينات حساسة ومتخصصه وبتطبيق هذه الدراسة يكون لها مردود أقتصادي مؤثر في إنتاج البكتريين والانتيجينات المختلفة لميكروب الهيموفيليس بارا جالينيرم.