STUDIES ON *PASTEURELIA MULTOCIDA*

I. INHIBITORY ACTION OF BLOOD ON THE GROWTH OF *PAST. MULTOCIDA*

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Many years have elapsed since blood agar was introduced for the isolation, subculture of strains and hemolytic properties of *Past. multocida*. And now, blood agar is generally considered to be one of the most suitable media for cultivating the organisms. Certainly, the organisms colonize indefinitely or do not even colonize at all on the nutrient agar medium.

Firstly, we should be sure that antibodies against the organisms do not exist in the blood which will be used to enrich the nutrient agar medium, but heretofore little attention has been paid to this point. Recently, LITTLE and LYON¹⁾ reported that one hundred and seventy three samples of normal horse serum were examined with respect to agglutinin for type 1 *Past. multocida*. Agglutinin was demonstrated in 24% of the serums tested, and it was said that such blood would reduce the virulence of the Pasteurella organisms and that it was not suitable for the cultivation of Pasteurella organisms. Moreover it is recognized, at present, that there will be many carriers of this organism where Pasteurellosis prevails. Therefore it is necessary to examine the blood before use.

Secondly, we must consider whether the blood contains any bactericidal constituent for the organisms or not. It is well known that rat blood would kill *B. anthracis*, and that canine blood would kill typhoid organisms. Moreover ABDOOSH²⁾ reported that the blood of the rabbit or horse would inhibit the gonococcus organism, while HINGLAIS³⁾ recently reported that rat blood inhibited the growth of the human type of Tubercle bacillus, but we have not found any report concerning *Past. multocida*.

While we were studying the growth of *Past. multocida* on different media, we found that blood and serum agar had inhibited the growth of the organisms and that it is due to an inhibitory substance in the blood. The results of these studies are as follows.

MATERIALS AND METHODS

The observation of the inhibitory action of blood has been constantly carried out by using inoculation of the organisms on the plate medium. In these experiments, broth cultures were previously diluted with saline in serial dilution and then transferred to the plate. After a certain incubation, a comparison of the numbers and sizes of the colonies growing on the plates was made and observation on the manner of their growth was carried out.

All the media were routinely prepared from meat-infusion-broth used as a basic culture and the pH was adjusted by 10% Na₂CO₃, 10% NaOH and 4% NaOH solutions, respectively. The bloods were collected aseptically from goats, horses, cattle, swine, rabbits and chickens, and then added to the nutrient agar medium in the ratio of 1×15.

All of the strains used for testing had been stocked in our laboratory and included 8 swine strains (S), 12 fowls strains (F) and 1 duck strain (D), only the S3 strain had just been isolated, the others were successively transferred to the blood agar and stocked in the refrigerator for over one year. According to Dr. CARTER's serological examinations^{4,5)} of the above strains, all were type A except the strain S8 which showed a weak type C reaction.

After seeding the culture from the slant into the broth and incubating for 20-24 hours at 37°C, it was diluted with saline from 10^{-1} to 10^{-6} and then from each dilution one loop (3mm) was transferred to an agar plate. Afterwards the plates were settled in the incubator and cultivated for 38-48 hours.

RESULTS

(1) The inhibitory action of goat and horse blood on the strains F2 and F6

At first, we observed that the growth of Pasteurella organisms on blood agar was poor in comparison with that on the nutrient agar medium. In order to determine whether this was due to the alkalis or to the goat blood, the following experiments were tried.

Table 1. A Comparison of the Growth of Strain F2 and F6 on Blood Agar with That on Nutrient Agar

Strain	Alkali		Blood agar				Nutrient agar			
			10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-3}	10^{-4}	10^{-5}	10^{-6}
F2	10% Na_2CO_3	3.6cc	oooooo	oooooo	oooooo	oooooo	oooooo	oooooo	oooooo	oooooo
		4.0	ooooo*	ooooo	ooooo	ooooo	oooooo	oooooo	oooooo	oooooo
		4.4	ooooo	ooooo	ooooo	ooooo	oooooo	ooooo*	oooooo	oooooo
		4.8	ooooo	ooooo	ooooo	ooooo	oooo*	oooo	oooo	oooo
		5.2	oooooo	ooooo	oooooo	oooooo	ooo	o	oo	oo
	10% NaOH	1.0	o	o	o	o	ooooo**	oooo**	oooo*	ooo
		1.2	o*	o**	o	oo	oooooo*	oooooo**	oooooo	oooo
		1.4	oo**	oo*	o*	oo	oooooo	oooooo	oooooo	oooooo
		1.6	oo	oo	oo	oo	oooo*	oooo*	oooooo	ooooo
		1.8	oo**	oo	oo	oo	oooooo	oooooo	oooooo	oooooo
	4% NaOH	4.2	oooo*	oooo	oooo	ooo	oooooo	oooooo	oooooo	oooooo
		4.6	oo**	oo**	ooo	oo	oooooo	oooooo	oooooo	oooooo
		5.0	oooo*	ooo	ooo	oo	oooooo*	oooooo*	oooooo	oooooo
		5.4	oooo	oo**	oo*	oo	oooooo*	oooooo*	oooooo*	oooooo
		5.8	ooooo*	ooooo	ooooo	ooooo	oooooo*	oooooo*	oooooo	oooooo
F6	10% Na_2CO_3	3.6	o	o	o	o	oooooo*	oooooo*	oooo*	oooo*
		4.0	o*	o	o	o	oooooo*	oooo*	oooo	ooooo
		4.4	oo*	o*	o*	o*	oooooo	oooooo*	oooo*	ooo
		4.8	o*	o**	o*	o	oooooo	oooo*	oooo	oooo
		5.2	o	o	o	o	oo	o	o	o
	10% NaOH	1.0	*	*	*	o	oooo*	oo	ooo	ooo
		1.2	****	***	o*	o	oooo*	oooo*	oo	ooo
		1.4	o**	o**	o		oooo*	ooo*	ooo	oooo
		1.6	*	**	o		oooo*	oooo	oooo	oooo
		1.8	*				oooo**	oooo	oooo	oooo
	4% NaOH	4.2	oo*	o**	o*	o	oooooo*	oooooo	oooo*	oooo
		4.6	*	*			oooooo*	oooooo*	oooooo*	oooooo
		5.0	o	o	o	o	oooo*	oooo*	oooo**	oooo
		5.4	o*	*			oooooo*	oooooo*	oooooo*	oooooo
		5.8	o	*			oooooo	oooooo	oooooo	oooooo

o shows normal growth. * shows poor growth.

Three media, adjusted by different alkali, were prepared, horse and goat blood were added and then the comparison of the growth of F2 with F6 was carried out about 7 times, including 5 times with goat blood and 2 times with horse blood. The broth culture was previously diluted as follows: 10^{-3} , 10^{-4} , 10^{-5} , 10^{-6} , and each of the diluted cultures was transferred to the medium for cultivation, the 10^{-6} dilution showed several colonies.

The results of 38 hours of cultivation are shown in Table 1, and we find that the growth of F2 and F6 was much more affected by the kind of alkalis than by their amount, whereas the growth was different for the two strains, that of F2 was better than that of F6. In short, the growth on blood agar was poorer than that on nutrient agar and the strains F2 and F6 were strongly inhibited by the goat and horse blood, and the result of 12-24 hours cultivation was also poorer than that of 48 hours cultivation.

(2) Comparison of the inhibitory action on the growth of *Past. multocida* by different animal bloods

Although horse and goat blood had inhibited the growth on the blood agar, the number of the strains used in these studies may have been too small so we tried all 19 strains with respect to their growth. All media were adjusted by 10% NaOH to pH 7.4 and cattle, goat, horse, rabbit, chicken and swine blood were added respectively, then the culture previously diluted to 10^{-2} , 10^{-4} , 10^{-6} was brought on each medium. The results are as in Table 2.

From Table 2, it can be seen that the inhibition by blood is very different between animal species and strains, the order of inhibition being as follows: Cattle and goat blood were strongest, swine and rabbit blood were rather strong, and chicken blood had no inhibitory affect on the organisms at all.

(3) Examination of agglutinin

As was suggested by LITTLE and LYON¹⁾, the normal blood might contain agglutinin against *Pasteurella* organisms, if so, it would influence the growth of *Pasteurella*. To

Table 2. A Comparison of the Inhibitory Action on the Growth of *Past. multocida* with Different Animal Blood

Strain	Cattle	Goat	Swine	Rabbit	Chicken	Nutrient agar
	10^{-2} 10^{-4} 10^{-6}	10^{-2} 10^{-4} 10^{-6}	10^{-2} 10^{-4} 10^{-6}	10^{-2} 10^{-4} 10^{-6}	10^{-2} 10^{-4} 10^{-6}	10^{-2} 10^{-4} 10^{-6}
F 1				(+)(+) +	+ + +	+ + +
F 2	(+)		(+)	(+)	+ + +	+ + +
F 3				(+)(+)	+ +	+ + +
F 4					+ + +	+ + +
F 5	(+)		(+)	+ (+)	+ + +	+ + +
F 6			(+)		+ + +	+ + +
F 7		(+)	+ (+)(+)	(+)	+ (+) +	+ + +
F 8					+ + +	+ + +
F 9					+ + +	+ + +
F10					+ +	(+) + +
F11				+ +	+ + +	+ + +
F12			(+)(+)	+ +	+ + +	(+)(+)(+)
S 1			(+)		+ (+)	(+)(+) +
S 2	+ + +	+ + +	+ + +	+ + +	+ + +	+ + +
S 3			+	(+)(+)	+ + +	(+)
S 4				(+)	+ (+) +	+ + +
S 7			(+)		+ + +	+ + +
S 8			(+)	(+)	+ + +	+ + +
D 1				+ (+) +	+ + +	+ + +

+ shows normal growth. (+) shows poor growth.

decide this question, we had to ascertain whether the goat and horse blood had any agglutinin or not. After separating the serum from the blood, it was diluted to 25X and 50X with saline, then examined by using the tube agglutinin test, but all results were negative; it could be said the factor of the inhibitory action did not come from serum agglutinin, although we had not used the HUGHES⁶⁾ buffer reaction test in this examination.

(4) The relation between blood components and its inhibitory action on the growth of *Past. multocida*

In view of examining which fraction of blood belongs to the growth-inhibitory substances, we prepared defibrinated blood, R.B.C. saline suspension, hemolized R.B.C. solution and serum. They were added to the nutrient agar medium which had been adjusted by 10% Na₂CO₃, 10% NaOH and 4% NaOH respectively. Afterwards the cultures of F2 and F6 strains were transferred onto these plate media.

As is shown in Table 3, except for F2, growing on 10% Na₂CO₃ media, all failed to grow on the blood and serum agar, while on the contrary, the growth on the medium containing blood corpuscles and their hemolized solution were very excellent as control. According to this experiment, we found that the factor of inhibitory action could certainly exist in the serum.

(5) The heat tolerance test of the inhibitory substance

For the purpose of observing whether the inhibitory substance could be the same as alexin, heat tolerance tests were carried out two times in view of the fact that alexin in serum can be destroyed by heat at 60°C. At first, we heated serum at 60°C for one hour, normal serum and whole blood were prepared, then the growth of *Pasteurella* organisms in these media were compared, the medium had been adjusted by 10% NaOH solution and the strain F2 was examined. In the next test, we compared the growth of *Pasteurella* organisms on the goat serum agar heated at 60°C for 30 minutes with fresh normal serum agar, the F3, F6 and F7 strains were examined and the media were adjusted by 10% NaOH

Table 3. The Relation between Blood Component and its Inhibitory Action on the Growth of *Past. multocida*

Blood component	Dilution	10% Na ₂ CO ₃		10% NaOH		4% NaOH	
		F2	F6	F2	F6	F2	F6
Whole blood	10 ⁻²	+		+		+	
	10 ⁻⁴	+					
	10 ⁻⁶	+					
R.B.C.	10 ⁻²	+	+	+	+	+	+
	10 ⁻⁴	+	+	+	+	+	+
	10 ⁻⁶	+	+	+	+	+	+
Hemolysis	10 ⁻²	+	+	+	+	+	+
	10 ⁻⁴	+	+	+	+	+	+
	10 ⁻⁶	+	+	+	+	+	+
Serum	10 ⁻²	+					
	10 ⁻⁴	+					
	10 ⁻⁶	+					
Nutrient agar	10 ⁻²	+	+	+	+	+	+
	10 ⁻⁴	+	+	+	+	+	+
	10 ⁻⁶	+	+	+	+	+	+

Table 4. The Inhibitory Action of Horse Serum heated at 60°C on the Growth of Strain F2

10% NaOH Solution	Blood component	Dilution			
		10 ⁻³	10 ⁻⁴	10 ⁻⁵	10 ⁻⁶
1.2cc	Blood				+
	Serum				
	60°C Serum	+	+	+	+
1.4cc	Nutr. agar	+	+	+	+
	Blood	(+)			
	Serum	+	+	+	+
1.6cc	60°C Serum	+	+	+	+
	Nutr. agar	+	+	+	+
1.8cc	Blood		(+)		
	Serum	+	+		
	60°C Serum	+	+	+	+
	Nutr. agar	+	+	+	+

Table 5. The Inhibitory Action of the Goat Serum heated at 60°C on the Growth of Strain F3, F6 and F7

Alkali	Strain	Serum			60°C Serum			Nutrient agar		
		10 ⁻²	10 ⁻⁴	10 ⁻⁶	10 ⁻²	10 ⁻⁴	10 ⁻⁶	10 ⁻²	10 ⁻⁴	10 ⁻⁶
10% Na ₂ CO ₃	F3				(+)	(+)	(+)	+	+	+
	F6				+			+	+	+
	F7	+						+	+	+
10% NaOH	F3							+	+	+
	F6				+			+	+	+
	F7							+	+	+
4% NaOH	F3				+			+	+	+
	F6				(+)			+	+	+
	F7				(+)			+	+	+

to pH 7.4. The results were as in Table 4 and 5.

As shown in Table 5 horse serum heated at 60°C for one hour and goat serum at 60°C for 30 minutes failed in having any inhibitory action on the growth of *Pasteurella* organisms.

From the above experiments, we know that the blood of horses, cattle, goats, swine and rabbits has an inhibitory action on the growth of *Past. multocida*, moreover, the inhibition was different in the cases of various animals and the grade of the inhibitory action was higher in horses, cattle, and lower in swine and rabbits, and absent in chickens. The inhibitory substance in the serum has nothing to do with agglutinin, and it was destroyed or reduce by heating at 60°C, thus it may be supposed to be similar to alexin.

DISCUSSION

The inhibitory action of blood on the growth of microorganisms has been well known for a long time and its real causative factor were, as reported recently by ABDOOSH, due to the existence of bactericidin (Alexin) in the serum, and as described by HINGLAIS, to the defect of growth enzyme in the blood. However in the present studies, the inhibitory action on the growth of *Pasteurella* organisms could be said to be due to Alexin.

The degree of sensitivity for the inhibitory action on the growth of the organisms was different among the strains, such as strong, weak or absent, but we have not examined as to whether the cause comes from natural or acquired properties being successively transfered on the agar medium. Moreover, we should consider that the virulence of the organisms would probably be affected by the inhibitory substance, therefore it is not suitable to use the blood agar for cultivation of *Past. multocida*.

SUMMARY

(1) The bloods of various animals have inhibitory action on the growth of *Past. multocida*.

(2) The degree of inhibitory action of the blood on the *Pasteuralla* organisms was higher in horses and cattle, and lower in swine and rabbits, and absent in chickens.

(3) The inhibitory substance in blood has nothing to do with agglutinin, and it was destroyed or reduced by heating at 60°C for 60 minutes, so it may be supposed to be similar to alexin.

(4) The degree of sensitivity for the inhibitory action of blood was also different among the strains.

(5) From the above experiments, blood or serum as nutrients for the cultivation of *Pasteurella* organisms should be avoided.

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REFERENCES

- 1) LITTLE, P.A. & B.M. LYON: *Amer. J. Vet. Res.*, **6**, 11 (1943).
- 2) ABDOOSH, Y.B.: *J. Hyg.*, **36**, 355 (1936).
- 3) HINGLAIS, H., HINGLAIS, M. & M. LANGLADE: *C. R. Soc. Biol.*, **150**, 1744 (1956); *Vet. Bull.*, **28**, 2 (1958).
- 4) CARTER, G.R.: *Amer. J. Vet. Res.*, **16**, 481 (1955).
- 5) CARTER, G.R.: *Amer. J. Vet. Res.*, **18**, 437 (1957).
- 6) HUGHES, T.P.: *J. Exp. Med.*, **51**, 225 (1930).

Pasteurella multocida に関する研究

I. 血液の *Past. multocida* に対する発育阻止作用

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余は *Past. multocida* に対する基礎的実験の途上、家畜の血液には発育阻止作用の存することを知り、これについて実験を行った。実験の結果を要約すると次の如くである。

1) 家畜の血液には *Past. multocida* の発育に対する阻止作用がある。

2) 血液の *Pasteurella* に対する発育阻止作用は馬、牛、羊の血液が最も強く、豚及び兎がこれらに次ぎ、鶏の血液にはその作用がない。

3) 血液の *Pasteurella* に対する発育阻止性物質は、血清中に存在し、60°C 30-60 分の加熱により減弱または消失する点より見て *Alexin* に類するものと考えられる凝集素とは無関係である。

4) 菌株により発育阻止作用に対する態度は一樣でなく、全然作用を受けないものがある。

5) 本実験の結果から見て、従来血液及び血清を *Past. multocida* の培養に使用していることは避けるべきであると思われる。