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INHIBITORY ACTION OF RABBIT SERUM ON THE GROWTH OF LEPTOSPIRES[1,2]

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ABSTRACT

Growth inhibitory effects of rabbit sera against Leptospires was tested by comparing the growth of 13 strains comprising 8 serotypes of Leptospires in the media incorporated with the sera from 20 rabbits.

In the pipetting inoculation test, only 4 out of 10 rabbit sera supported growth in the media inoculated with 10^{-3} of a 2 weeks culture and 3 out of 10 supported growth in those inoculated with 10^{-6} of the culture.

In the loop inoculation test, only 4 out of 10 rabbit sera supported growth of more than 6 among 12 strains of Leptospires tested.

Difference in growth was even observed among different strains of the same serotype in Korthof's medium incorporated with apparently non-inhibitory sera.

The individual sera which supported good growth were pooled as pooled serum 1. These which showed poor growth were pooled as pooled serum 2, and pooled serum 3 consisted of 10 randomly selected sera from rabbits. Of 12 strains of Leptospires studied, 11 strains grew in media incorporated with pooled serum 1, only 1 strain with pooled serum 2 and 4 strains with pooled serum 3. As shown in pooled serum 3, the nutritional value of non-inhibitory sera seemed apparently diminished or cancelled by the inhibitory action of the other sera incorporated in the same media.

It is generally recognized that animal serum is one of the most important growth factors for culture of Leptospires(1). Most of the media prescribed for coultivating Leptospires(2–7) require rabbit serum as the protein source and, it is stated by Boyd(8), that rabbit serum is most satisfactory among various animal sera studied. However, in addition to rabbit serum, horse(9, 10), bovine(11), sheep(12, 13) and pigeon(14) sera have also been recommended. Lately, it has been reported that the albumin of horse(15) and rabbit sera (16) supports the growth of Leptospires.

In cultivating Leptospires, one of the most striking features is the irregularity in growth pattern according to the kinds of media used. Shimizu *et al.*(17) and Ruth *et al.*(18–21) obtained different growth pictures of the organisms using different media.

In a previous report(22), the author

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stated that colony formation of *L. pomona* on Cox's solid medium varied when different pools of rabbit serum were used. In an experimental infection of sows with *L. pomona* (23), the author again observed that the organism ceased growth at an early stage in cultivation in Korthof's medium, and soon died.

The following experiment was designed to see the effect on the growth of Leptaspires in Korthof's medium when varied individual or pooled rabbit sera were incorporated in the media.

MATERIALS AND METHODS

The possible inhibitory effect of rabbit serum on Leptospires was tested by adding different individual and pooled sera to the Korthof's medium and examining for growth under the dark field 1–2 weeks after inoculating a small amount of the organism.

Media preparation:

The medium was prepared as described by Korthof, *i. e.*, 400 mg pepton*, 700 mg NaCl, 10 mg $NaHCO_3$, 20 mg KCl, 20 mg $CaCl_2$, 90 mg KH_2PO_4, 480 mg $Na_2HPO_4 \cdot 2H_2O$ were dissolved in 500 ml distilled water, heated at 100 C for 20 minutes, filtered when cool through Seitz filter (with ST-3, size L-6 Hercules filter pad) and then distributed in 27 ml or 45 ml amounts in 100 ml bottles and stored as the basal medium at 4 C. For the experiment, 10%, *i. e.*, 3 ml or 5 ml of individual or pooled serum was added in the Korthof's basal medium, inactivated at 56 C for 60 minutes and distributed in 1.8 or 2.0 ml amounts in the screw-capped tubes (1×12 cm).

Rabbit serum:

The rabbits used were supplied by local commercial animal dealers. Blood was collected by heart puncture and distributed in 10 ml amounts in Petri dishes. Serum was collected after standing at 37 C for several hours and centrifugation at 3000 rpm for 30 minutes. The individual sera were used on the day of preperation or within the next day and the pooled sera were stored for 2 to 3 weeks at 4 C before use with an equal amount of individual fresh sera.

Strains:

13 strains comprised of 8 serotypes *viz.*, *L. icterohemorrhagiae*, *L. canicola*, *L. autumnalis*, *L. hebdomadis*, *L. australis A.*, *L. grippotyphosa*, and *L. pyrogenes* were used in this study. They were maintained by subculturing in Korthof's medium with 10% individual serum and 2 week-cultures were used for inoculation in the experiment.

Inoculation:

The pipetting method and loop method were used for diluting Leptospires inocula.

Pipetting method—0.2 ml of Leptospires culture was pipetted serially to 5 tubes each containing 1.8 ml of Korthof's medium to make dilutions of 10^{-1} to 10^{-5} and 0.2 ml each of the 10^{-2} to 10^{-5} dilutions were added into a series of tubes containing 1.8 ml of the experimental media to make dilutions 10^{-3} to 10^{-6}.

Loop method—One 2 mm loopful of Leptospires culture in Korthof's medium was fished and inoculated into the tubes containing 2 ml of experimental media.

They were incubated for 1–2 weeks at 28 C after inoculation and examined for growth at 100× magnification under dark field illumination.

RESULTS

Examination for the growth of Leptospires at 1–2 weeks of incubation was done under the dark field illumination. The numbers of Leptospires seen in each 100× dark field was recorded as follows: less than 1 organism per field be −, 1 to 10, +; 11 to 30, ++; 31 to 100, +++; and more than 100, ++++ or +++++.

The numbers of Leptospires seen in the experimental media inoculated with 10^{-3} of Leptospires culture by the pipetting dilution method and loop method were both graded + right after inoculation. The

* The peptone used was "Kyokuto peptone" produced by Kyokuto Seiyaku Kogyo Co., Japan.

growth and survival time after 1–2 weeks of incubation differred according to the individual serum or the activity of the strains used. Therefore, a +++ growth seen at 1 to 2 weeks of incubation was considered to be evidence of multiplication.

TABLE I.

Growth of ***Leptospira pomona*** *in Korthof's medium two weeks after the inoculation of pipette diluted organisms.*

Rabbit no.	Sex	Weight (kg)	Strain	Growth in 10^{-3} to 10^{-6}
1	♂	2.4	Chugoku	10^{-3} –
2	♂	1.9	Chugoku	10^{-3} +
3	♀	3.1	Chugoku	10^{-3} –
			Ottawa	10^{-3} –
			Malcoln	10^{-3} –
			Kroeceburger	10^{-3} –
4	♂	2.5	Ottawa	10^{-4} ++++
			Kroeceburger	10^{-6} +++
5	♀	2.7	Chugoku	10^{-5} ++++
			Ottawa	10^{-6} +++
			Malcoln	10^{-4} ++
			Kroeceburger	10^{-3} –
6	♀	3.1	Chugoku	10^{-6} +++++
			Ottawa	10^{-6} +++++
			Malcoln	10^{-3} –
			Kroeceburger	10^{-3} –
7	♂	2.6	Chugoku	10^{-3} +++
			Ottawa	10^{-3} +
			Malcoln	10^{-3} –
			Kroeceburger	10^{-3} –
8	♂	2.7	Chugoku	10^{-3} +
			Ottawa	10^{-3} +++
			Malcoln	10^{-3} –
			Kroeceburger	10^{-3} –
9	♂	2.2	Chugoku	10^{-3} +++
			Ottawa	10^{-3} +
			Malcoln	10^{-3} –
			Kroeceburger	10^{-3} –
10	♀	3.8	Chugoku	10^{-3} –
			Ottawa	10^{-3} –
			Malcoln	10^{-3} –
			Kroeceburger	10^{-3} –

– Less than 1 organism per field
\+ 1-10 organisms per field
++ 11-30 organisms per field
+++ 31-100 organisms per field
++++ More than 100 organisms per field
+++++ The greatest number of organisms per field

TABLE II

Growth of Leptospires in Korthof's medium two weeks after loop inoculation.

Serotype	Strain	Rabbit no.	11	12	13	14	15	16	17	18	19	20	11-20
		Sex	♀	♂	♂	♂	♀	♂	♀	♂	♀	♀	
		B. W. (kg)	2.4	2.3	2.2	1.9	3.2	2.4	2.9	2.5	2.9	2.2	
L. icterohemorrhagiae	W		−	−	−	−	+++++	−	−	+++	+++++	+	−
L. canicola	Ut		−	+	+	+	+++++	+++	−	++++	+++++	++++	−
L. autumnalis	A		−	−	−	−	++	−	−	−	−	−	−
L. hebdomadis	B		−	−	−	+	+++++	+++	+++	++++	++++	+++++	++
L. australis A	C		+	+	+	+	+++++	++++	+++++	+++++	+++++	+++++	+
L. pyrogenes	S		−	−	+	+	+++++	+++	+++	++++	+++++	+++++	+
L. pyrogenes	Oki		−	−	−	−	+++++	−	−	+++	+	++++	−
L. grippotyphosa	BO		−	−	−	−	+++++	−	−	−	−	+++	−
L. grippotyphosa	MV		++	−	−	+++++	+++++	−	+++++	+++++	+++++	+++++	+++++
L. pomona	Chugoku		−	−	−	+	+++++	−	−	−	++++	+++++	−
L. pomona	Ottawa		−	+	−	+	+++++	−	−	−	+	+++++	−
L. pomona	Malcoln		−	−	−	−	+++	−	−	−	+++	+++++	−
Number of strains in the growth ## to ###			0	0	0	1	11	4	4	7	8	10	1

The growth of 4 strains of *L. pomona* seen in Korthof's medium incorporated with serum from Rabbit Nos. 1–10, diluted by pipette, and incubated 2 weeks is shown in TABLE I. The growth of the Leptospires was influenced greatly by the individual sera used. Also, each strain reacted differently in the same serum-incorporated medium. As seen in the TABLE, among 10 rabbits studied, there were 6 sera which supported growth up to 10^{-3}, 3 sera up to 10^{-6}, and 4 sera not even at 10^{-3}. Different growth was observed among 4 strains even in the medium incorporated with the same individual serum. The number of Leptospires seen right after the inoculation of 10^{-1} culture was ++++. In the medium incorporated with an inhibitory individual serum, the number of Leptospires remained about the same at the first week and then abruptly diminished after 2 weeks and many showed only less than + of the organisms left. In the medium incorporated with non-inhibitory serum, more than ++++ organisms were supported even after 4—6 weeks.

The growth at 2 weeks, seen in the Korthof's medium incorporated with Rabbit Nos. 11—20 individual serum, inoculated with 12 strains (8 serotypes) of Leptospires by loop method is shown in TABLE II. Results of this series were similar to the first, *i. e.*, only 4 (Nos. 15, 18, 19 and 20) out of 10 rabbit sera supported more than +++ growth of more than 6 strains, the others less than 4 strains and 3 none of the 12 strains of Leptospires studied. Generally, the growth at 2 weeks of incubation was better than at 1 week of incubation. Maximum growth was obtained (+++++) at 2 weeks of incubation when non-inhibitory sera were used, whereas irregular growth was noted with the inhibitory sera and some strains of Leptospires diminished in number at 2 weeks of incubation. Significant differences in growth among strains of the same serotype were seen more frequently in *L. pyrogenes*, *L. grippotyphosa* and *L. pomona*.

The growth of 12 strains of Leptospires inoculated by loop method into the Korthof's medium incorporated with non-inhibitory (Pool 1), inhibitory (Pool 2) and randomly selected (Pool 3) pooled sera from Nos.

TABLE III

A comparison of pooled sera for the growth of Leptospires

Serotype	Strain	Pool		
		1	2	3
L. icterohemorrhagiae	W	+++++	−	−
L. canicola	Ut	+++++	−	+
L. autumnalis	A	+++++	−	−
L. hebdomadis	B	+++++	+	++
L. australis A	C	+++++	−	++++
L. pyrogenes	S	+++++	−	+++
L. pyrogenes	Oki	++++	−	−
L. grippotyphosa	BO	+++	−	+
L. grippotyphosa	MV	+++++	+++	+++
L. pomona	Chugoku	+++	−	−
L. pomona	Ottawa	+++++	+	++++
L. pomona	Malcoln	−	−	−
Number of strains in the growth ## to ###		11	1	4

11—20 rabbits is shown in TABLE III. As seen in the TABLE, the growth of the 12 strains of Leptospires differred markedly with the pools used. Pool 1 showed the best and pool 2 the worst results. They were expressed as such according to the number of strains showing more than +++ growth. It should be noted that pool 3 was prepared by mixing equal amounts of 10 rabbit sera and contained non-inhibitory as well as inhibitory sera at the ratio of 4:6, *i.e.*, non-inhibitory sera comprised 4% of the whole medium and its supportive value might have been cancelled to some extent by the inhibitory sera.

No relation was found between the growth of Leptospires and the sera from rabbits of different sex and body weight.

DISCUSSION

Complete or incomplete inhibitory effects were observed in some individual sera incorporated in Korthof's media when minute amount of Leptospires were inoculated either by pipetting dilution or loop methods. It was further noted that the nutritional value of non-inhibitory sera was diminished by inhibitory ones when they were pooled together. Although no experiments were done to explore the factor responsible for the inhibitory action, it seemed sufficient to explain the irregular growth patterns encountered in many laboratories to be due to the presence of growth inhibitory effects among high percentages of rabbit sera used. Comparing Korthof's medium and chicken embryo tissue culture, a Japanese worker(24) reported no growth of Leptospires in Korthof's medium when minute amount of the organism was inoculated.

On the other hand, different growth was observed among different strains of the same serotype of Leptospires even in the same serum incorporated medium. This difference in growth is thought to be due to differences in activity of Leptospires themselves.

It has been a common practice either for seed culture maintenance or for agglutination lysis antigen preparation to inoculate 1/10 volume of the medium of Leptospires culture. By this method, the growth inhibitory effect of the serum is not significant. It is not quite clear as yet why there is growth when the inoculum is large and no growth when the inoculum is small. Many strains have been lost because of their poor growth.

Pipetting dilution method was found to be superior to the loop dilution method in obtaining a higher dilution of Leptospires culture for inoculation. However, it should be remembered that the pipetting dilution method might do harm to the activity of Leptospires. It is known that pipetting and shaking do influence the viability of even spores(25) in addition to their well known effects on many bacteria. The pipetting dilution method might best be used for the purpose of screening the suitability of sera to be used by loop method and for reducing the incubation and observation period of culture.

Yanagawa *et al.* (26) used a chamber for comparing the Leptospiral growth. It is necessary to use a chamber when comparing the growth in the media inoculated with large amount of leptospires because dark field examination is not suitable for the purpose. However, dark field examination sufficed the purposes of the present experiment because minute amounts of inoculum were used. Note that accuracy under dark field depends upon clarity of medium, and Krothof's does tend to become turbid upon heating at 100 C or 121 C.

In the present study, the results were read at 2 weeks of incubation. It might be feared that 2 weeks incubation is too short and the growth might not have attained its peak by this time. However, the experience at the beginning of this experiment showed better growth at 2 weeks than at 3 weeks and, in fact, little growth was observed after the 3rd week in the inhibitory sera incorporated medium. It was observed that growth attained its

peak at 1 week of incubation in the non-inhibitory sera incorporated media.

Preliminary tests incorporating the 3 pools of sera used in this study into Stuart's, Cox's and Fletcher's (Difco) media showed results similar to those with Korthof's medium when inoculated with the same 12 strains of Leptospires.

From the present study, it is obvious that consideration of serum inhibitory action is of great importance in isolating Leptospires from animals.

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