STUDIES ON LEPTOSPIRAL COLONY FORMATION IN BOTTLE CULTURE: CONDITIONS FAVORING *LEPTOSPIRA ICTEROHAEMORRHAGIAE* COLONY FORMATION

Takao FUJIKURA

First Research Division, National Institute of Animal Health, Kodaira, Tokyo

(Received for Publication August 7, 1966)

Leptospirae grow in a variety of media, including Korthof's, Fletcher's, Noguchi's, Stuart's, and Hindle's medium²⁾. The liquid or semi-solid properties of these media, however, make them unsuitable for certain leptospiral experiments. Reports in the last decade have described the rest phase of the organism¹³⁾, the effect of oxygen tension on leptospiral growth⁸⁾, and two morphological types of leptospiral colony formation on Cox's agar medium^{4,14)}. These studies suggested the development of a simplified colony assay for various purposes^{6,11)}. It soon became apparent, however, that a certain aspect of the dynamics of normal colony formation had to be clarified before the results of experiments were appreciated⁷⁾. The technique employed in the present investigation was one developed by the another.

MATERIALS AND METHODS

Leptospira strain: The strain of *Leptospira icterohaemorrhagiae* Weil mostly used in this study had undergone 8 successive passages in Korthof's medium. It was kindly supplied by Dr. M. KITAOKA of the WHO/FAO Leptospirosis Reference Laboratory at the National Institute of Health, Tokyo, Japan. The leptospirae used in certain experiments had, after several passages in Korthof's medium, been passed once in Cox's agar medium⁴⁾ and then once again in Korthof's medium. Seed leptospiral stock was propagated at 33°C for 7 days in screw-capped test tubes containing Korthof's medium. All dilutions of leptospirae were made in 0.2% Difco-tryptose phosphate broth (TPB) solution.

Culture media: Almost the same components as those described previously²⁾ were used to prepare Korthof's medium. The solid medium used consisted of Cox's solution containing 0.2 w/v% of TPB solution, 1.0% of Difco-bacto agar, and 10% of slightly hemolyzed normal rabbit serum. All sera employed in media had been tested individually for the presence of antibody against *L. icterohaemorrhagiae* both by agglutination reaction and by the colony inhibition neutralization test. In the latter test, approximately 100 colony forming units (CFU) of Weil strain leptospirae per 0.1 ml were incubated at room temperature for an hour with an equal volume of a 1:4 dilution of serum. Neutralization mixtures were then inoculated, in 0.2 ml volumes, into four assay media for colony formation. A log neutralization index of greater than 0.3 was considered to indicate the presence of antibody.

Techniques of colony formation: Rubber-stoppered prescription bottles 20, 60, and 200 ml in capacity and tape-sealed Petri dishes 60 and 90 mm in diameter were used. Seed leptospiral stock containing approximately 10⁸ CFU was diluted serially with 0.2% TPB solution so that the resulting dilutions might contain approximately 30~100 CFU

per 0.2 ml. Within 30 minutes after dilution 0.2 ml of each dilution was routinely inoculated into 4 or 5 assay bottles or plates. Cox's aqueous solution was sterilized by autoclaving at 121°C for 20 minutes and then cooled to $42\pm 1^\circ\text{C}$ in water bath. Normal rabbit serum (NRS) was then added to the solution to a final concentration of 10%. Cox's solution was used immediately after preparation or after storage at -20 to -40°C for several weeks. At the time of use, the solution was brought to a temperature of about 100°C . After addition of NRS at $42\pm 1^\circ\text{C}$, it was poured directly into the culture bottles or plates. The slow flow rate of the solution made it possible, after some practice, to keep the amount poured into a given vessel constant, i.e., 2 ml for a 20-ml bottle, 5 ml for a 60-ml bottle or plate, and 20 ml for a 200-ml bottle or 90-mm plate. Then the medium was added to the culture vessels, which were rotated gently for thorough distribution of the inoculum all over the medium, and which were allowed to solidify at a horizontal position. Then the vessels were immediately inverted to dry and placed on a horizontal shelf in an incubator at 33°C . The horizontal position of the shelf seems to be important, because the fluid barely covers the surface of the layer. A warm room or incubator is also necessary, since an ordinary laboratory incubator vibrates slightly when opened and shut. This technique possibly results in a greater dispersion of progeny leptospirae in colony assay. In colony assay, colonies grown in the bottles or plates incubated at 33°C were counted on the 7th to the 21st day of incubation. The colony count generally reached its maximum on the 17th day in the case of the Weil strain. In counting, a bottle or plate was placed on a bacterial colony counter and examined with the aid of a magnifying glass.

Preparation of antiserum: Four antibody-free rabbits were immunized with undiluted stock culture fluid of the Weil strain. Each animal was inoculated intravenously with 10^8 to 10^9 viable leptospirae five to six times at 3 or 4 days' intervals. All the animals were bled and sera pooled. The sera were stored at -20°C and inactivated at 56°C for 30 minutes prior to use.

Fifty per cent culture dose (CD_{50}) of leptospirae in Korthof's medium: A Petroff-Hauser bacteria counter gives results rapidly in quantitative measurement of viable leptospirae. The disadvantages of this method are that there is no unequivocal way of distinguishing dead cells from living ones, that the method is not applicable to an extremely high or low leptospiral population, and that it may cause a considerable strain of the eye if many counts are made. Titration by the terminal dilution method can be made more accurately by cultivating 5 samples, in 0.2 ml volumes, of each leptospiral seed dilution in 5 replicated screw-capped test tubes containing 10 ml each of Korthof's medium. For one or more dilutions, there will be some positive and negative cultures, and it will be possible to estimate by interpolation the dilution that will give 50% positive and 50% negative results of culture.

RESULTS

Experiments on dependence of colony formation by seeding method: These experiments were designed to establish techniques of the seeding method for the cultivation of the Weil strain. In appraising the accuracy of this method, several points must be borne in mind.

In this method, approximately 100 CFU of the strain were seeded in systems, one consisting of 20-ml, 60-ml, and 200-ml bottles and the other of 60-mm and 90-mm Petri dishes. Colony-forming efficiency was compared between the two systems (Chart 1). It was found that 60-ml or 200-ml bottle was more useful for the colony formation than

Chart 1. Number of Colonies of the Weil Strain in Various Culture Vessels at Different Days of Cultivation

Primary colonies appeared on the 8th day of cultivation and reached a plateau between 10 to 15 days. (X), 60-ml bottle; (O), 200-ml bottle; x, 20-ml bottle; Δ, 60-mm Petri dish; □, 90-mm Petri dish; ●, Seed leptospirae were spread on the surface of the medium in 90-mm Petri dishes. Each point represents the mean of replicated bottles or Petri dishes. The arrow points to the first appearance of secondary colonies.

Table 1. Leptospiral Colony Formation in Cox's Medium Containing Various Concentrations of Agar

% of agar	Leptospiral colony formation
0.5	(+)
1.0	+
2.0	+
3.0	+
5.0	—

(+): indefinite form of colony formation.

+: definite form of colony formation.

—: no colony formation.

Table 2. Leptospiral Colony Formation in Various Quantities of Cox's Medium

Quantity of Cox's medium (ml)	Layer thickness (mm)	Leptospiral suspended fluid seeded (ml)	No. of colonies formed*	No. of colonies per ml of medium
2.5	1.0	0.025	not formed	—
5.0	2.0	0.05	82	16.4
10.0	2.8	0.10	159	15.9
15.0	5.0	0.15	188	12.5
20.0	7.8	0.20	233	16.7
30.0	10.5	0.30	266	8.9

* Colony count on the 14th day of incubation.

The number of colonies is represented by the average of 4 replicated 200-ml bottles.

any Petri dish. For practical purposes, 200-ml bottles were employed throughout the experiments. Some workers^{3-6, 11, 14} have used another method in which culture is spread on the surface of the solid layer in a plate. Comparative studies revealed that this method was useful for colony formation, but that it sometimes failed to grow such number of colonies as was obtained by the bottle culture method. The results obtained from the experiments on seeding in various concentrations of agar medium (0.5, 1.0, 2.0, 3.0, and 5.0%) and in different quantities of the medium (from 2.5 to 30.0 ml) are presented in Tables 1 and 2. When the agar medium was used, the colony-forming efficiency in the bottle was dependent on the agar concentration and quantity of the medium. The agar concentration of 1.0% and the agar quantity of 5~20 ml were found to bring about superior results.

Effect of heat on seeding procedures: In an attempt to determine the effect of temperature on leptospirae during seeding, seed-agar mixtures were heated at several temperatures for various periods of time. Then the samples were withdrawn from the water bath and cultivations were made for

Table 3. Effect of Heat on Leptospirae

Temperature of heating (°C)	Time in minutes of heating		
	10	20	30
50	2.5*	2.5	2.5
47	7.5	6.0	4.5
44	7.8	7.8	6.0
41	7.8	7.8	7.5
Not heated	7.8	7.8	7.8

* Log₁₀ CD₅₀/ml of Korthof's medium.

titrations. Titrations were performed in 5 test tubes of replicated Korthof's medium. The survival ratios are shown in Table 3. Treatment at 41 to 44°C induced only slight inhibition, and no temperature exceeding 50°C gave rise to any colonies even when it lasted for only 10 minutes. Leptospiral seeding at $42\pm 1^\circ\text{C}$ within 20 minutes was chosen throughout the work.

Investigation of seeding in three layers: Prior to experiments, including quantitative and morphological observations, it was necessary to examine colony formation in various depths of the medium. Depth would initiate some demonstrable difference in the colony count with some morphological changes, since it is conceivable that the same oxygen tension might be found in a layer of the medium and any other surrounding condition of trapped leptospirae.

In an experiment, leptospirae were seeded at three different depths of the medium in the following manner: (1) A 5-ml seeding layer was overlaid upon a 15-ml nutrient agar base previously solidified in a 200-ml bottle. (2) A 5-ml seeding layer was sandwiched between two 7.5-ml nutrient agar bases. (3) A 15-ml nutrient agar base was overlaid upon a 5-ml seeding layer previously solidified. In this procedure, 0.2-ml of each inoculum was transferred to the seeding medium and mixed thoroughly by rotation of the bottle. When the medium became solid, the organisms were trapped in the gel at various depths of the inner medium. An assumption has been made that as each organism multiplies, a macroscopically visible mass of organisms may develop, thus one organism giving rise to one colony. The numbers of colonies obtained from the three medium layers are given in Chart 2. There were comparable colony counts in the three

Chart 2. Colony Formation at Various Depths of Layers in Cox's Medium

experiments. Although there may have been a slight excess of count in the sandwiched layer (33.5 ± 3.5), the colony count was reduced in the bottom-most layer (29.5 ± 6.5). It was 24.7 ± 8.1 in the top-most layer. The standard deviations of the means between the 4 replicated bottles in each of the three series of experiments overlapped each other, and these data may not be significant. This decrease in colony count in the top-most and bottom-most seeding layers may possibly be explained by the presence of such micro-aerophilic range as less suitable for leptospiral growth. These experiment suggest that the depth of seeding cultivation may have little significant effect on leptospiral colony formation. No striking changes were observed in the time of appearance or the morphology of colonies.

Time of appearance of colonies: It is obvious that colonies can be recognized microscopically only when they exceed a certain minimum size. Those colonies which are below the visibility threshold when the layer is magnified 6~7 times are called "infracolonyes"⁹. No colonies will be seen for some time after cultivation. Then

colonies will appear, increasing gradually in number and size. Eventually a plateau is reached, when the number of colonies developing from the initial inoculum, or that of "primary colonies," remains constant. At some time, "secondary colonies" may become visible. These colonies are derived not from the primary colonies, but from the newly grown progeny of leptospirae diffusing away from the primary colonies. The rate at which colonies emerged into visibility was determined in the following experiment with serially diluted seeds. Chart 3 shows a strict linearity of the response obtained over a considerable range when tenfold dilutions of the Weil strain suspension had been seeded in 4 replicated 200-ml bottles.

The first primary colonies emerged on the 8th day. After the 11th day there was no further increase in colony count. On the 15th day, however, secondary colonies appeared and increased in number with the advance in incubation. They were at first readily distinguished from primary colonies by their mother colonies (Fig. 1). Later, these features became less conspicuous. Thus, at 14 days

Chart 3. Proportionality between the Amount Weil Strain *Leptospirae* Cultivated and Colony Count

Results from four experiments in which more than one leptospiral dilutions were evaluated are presented. The sign represents the mean colony count obtained in a 200-ml bottle on the 14th day of cultivation.

Chart 4. Frequency of Distribution of Colonies

The histogram represents experimentally determined colony counts. The calculated normal distribution for independently distributed leptospirae in suspension is indicated by a solid line.

of incubation the largest number of primary colonies was reached and confusion with secondary colonies avoided. So that this period of incubation was adopted for the assay.

Uniformity of the assay system: A total of 360 replicated bottles which had been inoculated with an equal amount of a leptospiral suspension of low titer were used to determine the distribution of colony counts. This was to compare the theoretical with the normal distribution as calculated from the mean value obtained. The experimental and the theoretically determined points were plotted in Chart 4. The mean was 99.3 and the standard deviation of the experimental results 19.7. A χ^2 of 6.48 has a probability of about 0.9.

There is thus no reason for postulating any variability in the assay system other than that expected from random sampling.

Sensitivity of the colony method in comparison with the 50% culture dose in Korthof's method: To get a basis of comparison, titers

Table 4. Comparative Titers of the Weil Strain Determined by Colony Counting and the Korthof's Medium CD_{50} Method

Expt. No.*	Leptospiral dilution	Colony count (average count in parentheses)	CFU/ml	No. of positive cultures in Korthof's medium	CD_{50} /ml	Ratio of CFU/ml to CD_{50} /ml
1	10^{-5}	332, 305, 314, 328 (319.8)	2.54×10^8	5/5†	3.16×10^8	1:1.24
	10^{-6}	27, 36, 29, 27, 19 (27.6)		3/5		
	10^{-7}	1, 2, 2, 6, 0 (2.2)		2/5		
	10^{-8}	1, 0, 0, 0, 0 (0.2)		0/5		
2	10^{-6}	78, 54, 46, 68 (61.5)	5.85×10^8	5/5	6.03×10^8	1:1.03
	10^{-7}	5, 6, 6, 7, 8 (6.4)		5/5		
	10^{-8}	0, 0, 0, 0, 2 (0.4)		2/5		

* Each experiment was performed independently. Each culture was inoculated with 0.1 ml of leptospiral dilution.

† Numerator=No. of positive culture test tubes; Denominator=No. of culture test tubes inoculated.

of some samples of the stock culture were calculated by the 50% culture dose (CD_{50}) in Korthof's medium at the terminal dilution of cultivation. The same samples were titrated by the colony method at the same time. The results are given in Table 4. The ratio of CFU/ml to CD_{50} in Korthof's medium ranged from 1:1.03 to 1:1.24.

Colony inhibition assay of antiserum: The antiserum assay based on the inhibition of colony formation of the Weil strain probably has not been used by any other worker. Serially diluted suspensions of seed leptospirae containing $10 \sim 10^8$ CFU were mixed with equal volumes of serially diluted antiserum ($100,000 \times$ in agglutination titer) and incubated at 33°C for 60 minutes. The mixtures in 0.2 ml amounts were transferred to bottles for colony formation. In most cases, three replicated bottles were used. The results obtained on the 14th day of cultivation, in the presence or absence of antiserum, are shown in Table 5. The colonies in the antiserum group were small in number. Colonies had become visible in most of the untreated control cultures by the 8th day, but the treated cultures showed a very few colonies. In some of the treated cultures colonies were skewed in smaller size; in others they were comparable to those of the untreated. After the 11th day of cultivation, there was no further emergence of visible colonies.

Table 5. Effect of Antiserum on Colony Formation of the Weil Strain of *L. icterohaemorrhagiae*

Antiserum dilution*	Reciprocal $\log_{10}$ of leptospiral dilution								
	1.0	2.0	3.0	4.0	5.0	6.0	7.0	8.0	9.0
Undiluted	0	0	0	0	0	0	0	0	0
1:10	0	0	0	0	0	0	0	0	0
1:20	+	0	0	0	0	0	0	0	0
1:40	+	+	0	0	0	0	0	0	0
1:80	+	+	0	0	0	0	0	0	0
1:100	+	+	+	0	0	0	0	0	0
1:1,000	+	+	+	+	0	0	0	0	0
1:10,000	+	+	+	+	+	0	0	0	0
1:100,000	+	+	+	+	+	±	±	±	±
Normal rabbit Serum	+	+	+	+	+	+	+	+	+

* Tenfold and partially twofold serial dilutions were made from undiluted normal rabbit serum. For the procedure, see the text. 0: no detectable colony; +: colony formation observed; ±: skewed forms of colonies observed.

These results indicate that leptospirae were neutralized proportionally and that in the case of seeding with leptospirae of a relatively small number of CFU colony formation was prevented by a homologous anti-serum. No serological examination, however, was undertaken of the resultant colony strains in this assay.

Observations on the two types of colonies: During the course of seed cultivation, two types of colonies were observed^{4,14}). COX and LARSON⁴) also recognized two types of colonies, "the first being smaller in diameter and more opaque, and the second being quite translucent, of greater diameter, and veil-like in appearance." The two types of colonies observed herein were largely similar to those described by Cox (Fig. 2). In this paper the term "C type" is used to indicate a small, compactly formed colony (Cox's type 1), and the term "D type" a large, diffusely formed one (Cox's type 2). Both types were examined for frequency of appearance in mixed colonies which had been obtained by leptospiral seeding in different manners at various days of culture. In Chart 5, the proportions of C-type to D-type colonies (C/D) are 1.0, 5.7, 26.2, and 3.1, at 3, 7, 10, and 28 days of culture respectively.

To confirm these findings, it was necessary to attest that these colonies were derived from a single cell, i.e., that they were clones. To obtain pure lines of both types of clones from subcultures, both types of colonies were isolated and recultivated separately. For subculture, the colonies required were picked up with a fine capillary pipette, transferred to Korthof's medium, and then recultivated for colony formation. When this was done, colonies of one type generally gave rise to colonies of the same type alone. Fig. 3 illustrates the isolation of pure clones of C and D types from a single colony.

Miscellaneous observations: The number and size of colonies were examined at different times during cultivation. At early times, colonies found were small in size and increased in total number. It was assumed that at any given time within the interval of 11 to 14 days, there might be a constant number of primary colonies composed, at varying proportions, of those still in the infracolony stage and visible ones. The range of diameter, such as 0~1 mm, 1~2 mm, and 2~3 mm, was measured in 3 replicated bottles at each of the time intervals tested. The rate of increase in diameter seemed independent of the size of the colony under consideration at a given time and of the time when a given diameter was reached. Bottles incubated for more than 14 days were increasingly difficult to analyze. The distribution of size was no longer normal. The colonies of C and D types were 2.3 ± 0.8 mm and 4.3 ± 0.8 mm, respectively, in diameter just before the 15th day of cultivation, or at the secondary-colony emergence.

In order to set proper physical conditions for the strain, cotton-plugged bottles were placed in a jar, from which the air had been evacuated by a vacuum pump and replaced with CO₂ from a manometer. The results obtained from these cotton-plugged bottles were not significant, as compared with those from rubber-stoppered ones.

Since benefits might result from early recognition and counting of colonies and precise observation of their morphological structures³), attempts were made to stain colonies with 0.1% dimethyl-*p*-phenylene diamine HCL (oxidase reagent). The examination was facilitated by flooding colonies. An addition of 0.05% 2,6-dichlorophenol in-

Chart 5. Different Proportions of C- and D-Type Colony Formation in 3-, 7-, 10-, and 28-day Culture Seed Leptospirae

dophenol to the medium also increased the visibility. Results of staining with methylene blue, phenol red, Wright's or Giemsa's solution were not remarkable enough to be described.

DISCUSSION

When the author studied leptospiral colony formation by the procedure of other workers^{4,14}), who had spread the seeds on the surface of medium layers in Petri dishes, he occasionally experienced such undesirable bacterial or fungal contamination of the plates as to lead to considerable loss of leptospiral culture. The possible causes of the contamination were unfixed cover dishes of plates and a prolonged period of incubation for more than 10 days. The evaporation of water from the medium during the incubation period was another unavoidable factor which made the medium hard and colonial emergence difficult. In addition, as recommended by some workers, the carbon dioxide supplying procedure for the assay system requires much regulated equipment¹⁵) and a limited number of dishes tested at one time. The procedure established in this study is considered to conquer most of these difficulties. In addition, it has some other advantage, with which the author isolated several leptospiral strains by cultivating terminal dilutions of contaminated Korthof's medium and of contaminated materials.

The growth of a single leptospiral colony seems to depend on the effects of a number of factors. The extent and velocity of spread of leptospirae in a medium, growth rate in the medium, and metabolic substances may probably be important. Furthermore, the concentration and the brand of agar used influence the growth of colonies. Finally, the reaction itself between leptospirae and normal serum may put limits on colonial growth. In this connection, several brands of agar and normal rabbit sera were examined before their use for medium.

The growth of a whole colony population was complicated by the fact that at a given time different sizes of colonies were present. Some of these colonies were so small as to be barely recognizable, and others were still hidden below the threshold of visibility. The latter were supposed to form an "infracolony" population¹¹). Consideration of the mean colony diameter or mean colony area based only on visible colonies can be misleading. It should be pointed out that the distribution of infracolonies is unknown, since it extrapolates below zero. A different approach would be required in a study of events taking place at the infracolony level. As shown in Table 4, when titration was performed in the same seeding suspension, values based on CFU were always lower than those of CD_{50} titrated in 5 replicated test tubes of Korthof's medium. Contrary to such complicated situation, it is surprising that a very simple relationship was found to describe the uniformity of the assay system expected from random sampling.

The isolation of pure clones of C and D types is illustrated in Fig. 3. These findings may conflict partially with those of other workers who had used different procedures¹). The results obtained from the other strain of *L. icterohaemorrhagiae*, including the relationships between the colonial morphology and the virulence for various animals⁵), body length, and heat sensitivity of the isolated clones, will be given in another paper to come¹⁶). More information is now needed about the colony dissociation and its relationship to the genetics of leptospirae. The colony formation of the other serotypes of leptospirae has been described by other workers^{3,14}). The author experienced colony formation when he studied *L. autumnalis* AB Akiyami A, *L. hebdomadis* Akiyami B, *L. australis* A Akiyami C, *L. pomona*, *L. andaman* CH 11, *L. semarang* RT 173, *L. biflexa* Urawa 3, and some others by the procedure described above. The findings on these serotypes will be

presented elsewhere. In their recent report, STALHEIM and WILSON¹² described the colonial morphology of leptospirae precisely on the basis of five different characters. In order to confirm these findings, further investigation is needed in addition to the present bottle culture method.

SUMMARY

When rubber-stoppered 200-ml prescription bottles were used, *Leptospira icterohaemorrhagiae* showed a more effective colony formation in Cox's medium than when Petri dishes were used. Approximately 30~100 colony forming units (CFU) of inocula (0.2 ml) were routinely put into 4 or 5 replicated bottles. Then 20 ml of Cox's medium, kept at $42\pm 1^{\circ}\text{C}$, was poured into the culture bottles. The bottles were gently rotated for thorough distribution of the inoculum throughout the medium, solidified horizontally, and incubated at 33°C for 14 to 21 days.

Colony counts were made depending on the concentration of agar, temperature of the medium at the seeding time, type of culture vessels, quantity of the medium poured in, and the depth of seeding in the medium.

Primary colonies were emerged into visibility during an incubation period of 8 to 14 days. In this period, colony counts gave a strict linearity of response within a wide range of seed leptospiral dilutions. The uniformity of the assay system of this method was expected from random sampling of colony distribution. The sensitivity of this assay system was 1.03 to 1.24 as expressed the ratio of CD_{50} in Korthof's medium to the colony count.

Two macroscopically distinguishable types of colonies were observed—a compactly small one (C type) and a diffusely large one (D type). To obtain pure lines of both types of clones, subcultures were done separately.

Suppression of colony formation by homologous antiserum was observed and reisolation of leptospirae from contaminated culture lines experienced.

Certain aspects of the dynamics of leptospiral colony formation were discussed along with the bottle culture method.

ACKNOWLEDGMENT

The author appreciates the interest in the present experiment taken by Dr. M. KITAOKA, of the WHO/FAO Leptospirosis Reference Laboratory at the National Institute of Health, Tokyo, Japan.

REFERENCES

- 1) ALEXANDER, A. D. (1963): Personal communication.
- 2) ALSTONE, J. M., and BROOM, J. C. (1958): Leptospirosis in Man and Animals, pp. 301~310., Edinburgh and London, E. and S. Livingstone Ltd.
- 3) ARMSTRONG, J. C., and GOLDBERG, H. R. (1960): *Amer. J. vet. Res.*, **21**, 311~315.
- 4) COX, C. D., and LARSON, A. D. (1957): *J. Bact.*, **73**, 587~589.
- 5) FAINE, S., and Van Der HOEDEN, J. (1964): *J. Bact.*, **88**, 1493~1496.
- 6) KIRSHNER, L., and GRAHAM, L. (1959): *Brit. J. exp. Path.*, **40**, 57~60.
- 7) LARSON, A. D., TREICK, R. W., EDWARDS, C. L., and COX, C. D. (1959): *J. Bact.*, **77**, 361~366.
- 8) LAWRENCE, J. J. (1951): *Aust. J. exp. Biol. med. Sci.*, **29**, 195~199.
- 9) LINDENMANN, J., and GRIFORD, C. E. (1963): *Virology*, **19**, 283~293.
- 10) PICKE, R. M., and SCHULZE, M. R. (1958): *J. Immunol.*, **81**, 172~175.

- 11) ROTH, E. E., LINDER, D., and ADAMS, W. V. (1961): *Amer. J. vet. Res.*, **22**, 308~312.
- 12) STALHEIM, O. H. V., and WILSON, J. B. (1963): *J. Bacteriol.*, **86**, 482~489.
- 13) WORAZ, H. (1954): *Zbl. Bakt., I. Abt. Orig.*, **160**, 613~628.
- 14) YANAGAWA, R., HIRAMUNE, T., and ISHII, S. (1959): *Jap. J. vet. Sci.*, **21**, 221~226.
- 15) YANAGAWA, R., HIRAMUNE, T., and FUJITA, J. (1963): *J. Bact.*, **85**, 875~880.

Addendum

- 16) FUJIKURA, T. (1966): *Jap. J. Microbiol.*, **10**, 79~83.

角びん内培養法によるレプトスピラ集落形成諸条件の検討

藤 倉 孝 夫

農林省家畜衛生試験場研究第一部

(昭和 41 年 8 月 7 日受付)

近年, Cox ら (1956), 梁川ら (1958) により, レプトスピラ (以下「レ」と略す) 集落形成については, 種々明らかにされてきた. これらの方法は, Cox 培地 (Difco-tryptose phosphate broth 0.2% に, 正常家免血清 10%, および Difco-bacto agar 1.0% を添加) を用いて, シャーレ内平板上に, 「レ」を接種し, 培養をおこなうものであるが, 7 ないし 21 日間の培養期間を通じて, 培地面よりの水分の蒸発, 汚染等のため, しばしば実験上損失をきたす場合にそうぐうした. そこで本研究においては, *Leptospira icterohaemorrhagiae* Weil 株を用い, 角びんを使用して, Cox 培地を添加し, 混釈培養により培養し, 種々検討した.

本培養方法による「レ」集落形成諸条件を検討し, 以下の成績をえた. すなわち, ゴム栓を施した 60 ml 容, 200 ml 容角びんを用い, Cox 培地それぞれ 5 ml, 20 ml 中に「レ」を接種し, $42 \pm 1^\circ\text{C}$ に保ちながら混釈培養し, 33°C で 21 日間培養したところ, 8 日

目に第 1 次集落が認められ, 15 日以降 21 日で第 2 次集落が発現した. 本培養方法は, 同時に比較した従来のシャーレ内平板培養法より良好な成績をあげ, 集落数分布は正規分布をしめし, 平均集落数 99.3 ± 19.7 における実測値および理論値との比較において, 本培養系の均一性が示唆された. また本培養方法による集落数計数による生菌数値と, Korthof 培地内限界希釈培養法による CD_{50} 値との比率は, 1:1.03 ないし 1:1.24 であった.

本試験により, 大 (D-type), 小 (C-type) 二型の「レ」集落の解離が観察され, それぞれの型の単個集落より得られた C- および D-substrain は, それぞれ C- および D-type の集落を均一に形成することが認められた.

これらの所見から, 本培養方法により, 「レ」集落が効率よく形成されることが明らかとなり, また, 「レ」集落に関する各種試験に応用しうることが示唆された.

EXPLANATION OF PLATE

- Fig. 1. Satellite distribution of secondary colonies around their mother primary colonies at 28 days of cultivation.
- Fig. 2. Colonies of C type (compactly formed, small) and D type (diffusely formed, large) dissociated in leptospirae-seeded Cox's medium at 10 days of cultivation.
- Fig. 3. Clones of C type and D type colonies isolated and cultivated separately. A: C type colony clone cultivation; B: D type colony clone cultivation. Photographs were taken at 10 days of cultivation.

Fig. 1

Fig. 2

Fig. 3