ENHANCEMENT OF LEPTOSPIRAL COLONIAL COUNT BY STAPHYLOCOCCAL CULTURE FILTRATE

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It has been known that staphylococci give rise to a interference or synergistic effect not only on staphylococci^{1,2,6)}, but also on some other kinds of pathogenic micro-organisms both *in vitro* and *in vivo* experimentally. Influenza virus, *Proteus*, and *Haemophilus* were known to be influenced by the synergistic effect of staphylococci.^{1,4,5)} In connection with the staphylococcal characteristics, the author examined the exalting effect of staphylococci on the leptospiral colonial formation, using especially the bottle cultivation method developed previously by the author³⁾. As a result, it was proved that staphylococci enhanced leptospiral colonial formation, and that there was an increase in the colonial count in the medium used.

MATERIALS AND METHODS

The *Staphylococcus aureus* S9-2 strain used in this study was isolated from a milk sample from a mastitic dairy cow. It was proved to be coagulase-positive and penicillin-resistant and show beta-type hemolysis.

The leptospiral strains used were *Leptospira icterohaemorrhagiae* Wijnberg, *L. autumnalis* AB Akiyami A, *L. hebdomadis* Akiyami B, *L. australis* A Akiyami C, *L. pomona* Pomona, *L. semarang* Veldrat S 173, and *L. biflexa* Urawa 3. They had been maintained in Korthof's medium. Ten- to 14-day-old culture stock was used for the seed cultivation.

Organisms of the staphylococcal strain were cultured in 100 ml of Difco-heart infusion broth over various periods. Then the culture was centrifuged at 7,000 rpm for 30 minutes and filtrated through a Seitz-EK filter. The resulting filtrate was used for experiment.

Seed leptospire, 0.2 ml of volume, were incubated and sensitized by mixing with 1.8 ml of staphylococcal culture filtrate at 37°C for an hour. The seed leptospire had been prepared by diluting the original seed culture fluid 10^{-6} to 10^{-8} so that 10 to 10^8 colony forming units (CFU) might be contained in 0.2 ml of seed culture fluid prior to the sensitization. The mixture in a 0.2-ml amount was seeded in 20 ml of Cox's agar medium which had been poured into a 200-ml prescription bottle at $42^{\circ}\pm 1^{\circ}\text{C}$. For cultivation, the bottle was kept at 33°C for 14 days.

Control experiments were set up in both Difco-heart infusion broth medium and Korthof's medium, for sensitization, so that results might be compared with those obtained from the staphylococcal culture filtrate.

The colonial count determined from an average number of colonies developed of five replicated bottles was referred to in the evaluation of the significance of the experiment.

RESULTS

Staphylococcal culture filtrates derived from 4-, 8-, 24-, 48-, and 72-hour broth

Table 1. Dilution Effect of Staphylococcal Culture Filtrate on Enhancement of Leptospiral Colonial Count

Leptospiral strain used ¹⁾	Dilution of staphylococcal culture filtrate ²⁾	Average number of leptospiral colonies $\pm$ standard deviation ³⁾	Percent of the enhancement of the colonial count
<i>L. biflexa</i> Urawa 3	1 : 1	48.7 $\pm$ 7.4	151
	1 : 2	48.3 $\pm$ 3.3	150
	1 : 4	48.0 $\pm$ 3.4	148
	1 : 8	47.8 $\pm$ 3.4	148
	1 : 16	47.7 $\pm$ 4.8	147
	1 : 32	26.7 $\pm$ 6.1	83
	Control	32.3 $\pm$ 5.3	100
<i>L. semarang</i> Veldrat S 173	1 : 1	35.7 $\pm$ 4.6	170
	1 : 2	30.0 $\pm$ 3.7	143
	1 : 4	27.4 $\pm$ 5.6	130
	1 : 8	26.3 $\pm$ 6.8	125
	1 : 16	22.3 $\pm$ 4.6	106
	1 : 32	21.3 $\pm$ 4.2	101
	Control	21.0 $\pm$ 4.5	100

1) Fourteen-day culture of leptospires was used for the colonial formation.

2) The filtrate was prepared from staphylococcal culture made at 37°C for 24 hours.

3) The average number of colonies and standard deviation were obtained from five replicated culture bottles in the same experiment.

cultures were mixed with the seed leptospires for sensitization at 37°C for an hour. Then the mixture was cultivated in Cox's agar medium for leptospiral colonial formation. It was observed that increased in number in 24- to 48-hour culture filtrates, as compared with the results obtained from other experiments. The colonial count showed an enhancement in 24-hour culture filtrate. It increased to approximately 150% of the control count in the case of the *L. biflexa* Urawa 3 strain and to 170% in that of the *L. semarang* Veldrat S 173 strain (Table 1). The enhancing effect of the filtrate generally decreased in 48- to 72-hour culture filtrates (Fig. 1).

The filtrate was examined for stability to the heating at 56°C. In this experiment, it was proved that the filtrate was stable to the heating and that the colonial enhancement occurred after heating at 56°C for 5 to 60 minutes. No significant decrease was seen in the effect of enhancement of the filtrate after the heating (Fig. 2).

Then dilutions of the filtrate were examined for any effect on the enhancing activity. Serial two-fold dilutions were made from the filtrate with broth medium. No remarkable dilution effect was seen in a 1 to 16 dilution of the filtrate in the experiment with the *L. biflexa* Urawa 3 strain. The effect was restricted in a 1 dilution in that with the *L. semarang* Veldrat S 173 strain. It was not evident in higher dilutions.

None of the other five pathogenic strains of *L. icterohaemorrhagiae*, *L. autumnalis* AB, *L. hebdomadis*, *L. australis* A, and *L. pomona* were revealed to have been influenced by the enhancing effect of the staphylococcal culture filtrate, so far as the present investigation is concerned.

DISCUSSIONS

It remains difficult to examine various kinds of organisms for the synergistic effect

Fig. 1 Enhanced Colonial Counts of *L. biflexa* Urawa 3 Strain in Staphylococcal Culture Filtrate Derived from Cultures at Various Hours of Cultivation

Fig. 2 Effect of Heating of Staphylococcal Culture Filtrate on Enhancement of Leptospiral Colonial Count

upon leptospire because of lack of any reliable method for the quantitative estimation of viable organisms of *Leptospira*. In this report, the author studied this effect by examining the leptospiral colonial formation by the bottle culture method, which had been proved to be available for experiments with leptospire³.

A possible explanation for the enhancing effect of staphylococcal culture filtrate on the leptospiral colonial formation will be given as follows: Since the period of sensitization of leptospire was relatively short in a mixture of these organisms and the staphylococcal culture filtrate, it might be impossible for leptospire to grow. It can probably be considered that the sensitization will make the less viable population of the seed leptospire in the mixture active to grow, that the clones of those which are fastidious to form colonies will be accelerated to appear by the sensitization, and that they will be enforced to grow to such an extent as to cross the visible threshold of colonial formation in the medium.

Some of the leptospiral clones in the stock culture fluid were in the stationary phase of growth. They were considered to be in a rather lower activity than those in the logarithmic phase of growth. It can not be expected for them to grow enough to be visible and form colonies unless they are sensitized by the staphylococcal culture filtrate. As indicated in the results obtained, the exalting effect upon the growth of leptospire was not shown significantly in the early phase of growth, or at 1 to 3 days of cultivation, though it was rather conspicuous in the stationary phase of growth.

SUMMARY

Leptospire showed an enhancement in colonial formation when they had been sensitized by the staphylococcal culture filtrate at 37°C for an hour. It was significantly observed that the colonial count was enhanced to 150 to 170 percent in the case of the *Leptospira biflexa* Urawa 3 and the *L. semarang* Veldrat S 173 strain, but it was not in the case of any pathogenic strain.

The activity of the staphylococcal culture filtrate to enhance the colonial formation would not be destroyed by the heating at 56°C for at least an hour. The filtrate also maintained this activity when diluted 1 to 16 in broth medium.

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ブドウ球菌培養濾液によるレプトスピラ集落数増強作用について

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ブドウ球菌は、同種の菌株の間ばかりでなく、*Proteus*, *Haemophilus*, *Influenza* ウイルスなど異種の微生物に対しても、培地中で増殖を促進すると、またこれらとの混合感染が、しばしば問題となってきた。本報告においては、さきに検討したレプトスピラ（以下「レ」と略す）の角びん内閉鎖系での集落形成法を応用し、ウシ乳房炎材料より分離した *Staphylococcus aureus* S9-2 株の heart infusion broth での培養濾液をもちい、「レ」集落におよぼす作用につき検討した。

上記のブドウ球菌株の 37°C, 18~24 時間培養液を Seitz EK で濾過し、無菌試験ののち、培養濾液として用いた。*Leptospira biflexa* Urawa 3 株の Korthof 培地 10~14 日培養原液を、0.2% tryptose phosphate broth で、10~1,000 集落形成単位を含むように希釈した。その 0.2ml と、培養濾液 1.8ml

を 37°C, 60 分感作後、ゴム栓を施した角びん内閉鎖系で、10% 正常ウサギ血清加 Cox 培地を用い、33°C で培養した。形成された「レ」集落数および、集落形態を、heart infusion broth, および、Korthof 培地感作の対照と比較したところ、150 ないし 170% の集落増の数加を認めた。集落形態については、いちじるしい所見は認められなかった。

この作用は、ブドウ球菌の培養時間と連関して変化した。24時間培養濾液が、最も本作用がいちじるしく、48時間以降次第に減少するようであった。また、培養濾液を、56°C で 1 時間加熱しても、この作用は低下することなく、1:8 ないし 1:16 希釈までこの作用は保持されていた。また *Leptospira semarang* Veldrat S173 株についても本作用を認めた。*L. icterohaemorrhagiae* ほか 4 株では、著明でなかった。