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TRANSLATION NO. 522

DATE: April 1961

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TRANSLATED FROM RUSSIAN

Translated at the
National Institutes of Health
Bethesda, Maryland

No. 4-13-61

From the Russian for
Dr. John F. Bell

Trudy Rostovskogo na Donu
gosudarstvennogo n.-i.
protivochnnogo in-ta, Vol. VI,
[Tularemia], 82-88, 1947.

Zhidkaya zheltchnaya sreda dlya vyrashchivaniya
kul'tur tulyaremi
Soobshcheniye III.. Sookhraneniye zhiznesposobnosti,
virulentnosti i agglyutinabil'nosti tulyaremiynykh
kul'tur pri khraneni i kh v zhidkoy zheltchnoy srede

A liquid egg yolk culture medium for growing Pasteurella
tularensis

Report III. Viability, virulence and agglutinability
of Pasteurella tularensis cultures preserved in the
liquid egg yolk culture medium

by

M. S. Drozhevskina

After some initial experiments which had demonstrated the advantages connected with the use of liquid egg yolk medium for growing Pasteurella tularensis, the author decided to determine whether protracted residence in the medium would affect the basic characteristics of the bacterium. Her first goal was to establish the extent to which the viability, virulence and agglutinability of the strains remain preserved.

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Viability of the tularemia agent in liquid egg yolk medium.
In result of the works of Khatenev and Levchenko, Knyazevskiy and Berdnikov, Somov, Tumanskiy, Usov, and others, the viability of the tularemia agent in objects belonging to the outer environment and in the bodies of dead animals under various storage conditions, is fairly well known.

A large number of studies have been devoted to the question of the extent to which the viability of Bact. tularensis is retained after long intervals in artificial culture media. For example, according to Khatenev and Levchenko, when it was kept at room temperature in sealed test tubes with coagulated egg yolk medium, Bact. tularensis survived without transplantation for 2 to 2.5 months. According to Pokrovskaya, it survived under these conditions for 1 to 2 months. Francis found in his tests with cysteine-agar that at 10°, Bact. tularensis remained viable without receding for periods ranging up to 3 months. In the coagulated egg yolk medium it remained viable even longer. O'Hara feels that in solid media, the cultures remain well preserved at room temperature for two weeks. If kept under these conditions for 3-4 weeks without reseeded, they no longer can be transplanted. Bact. tularensis was successfully preserved in a solid medium by Sinay for 26 days.

The results of the first series of experiments in which the viability of Bact. tularensis was to be tested without taking steps to prevent the medium from drying out (Report I), proved that in liquid egg yolk medium the cultures survive for long periods of time even under very unfavorable conditions.

In the 2nd series, steps were taken to keep the medium from drying. A series of test tubes containing liquid egg yolk medium were inoculated with strains Nos. 9, 14 and 16, the inoculation doses being 1 million bacterial units each. The test tubes were incubated for two days in the thermostat, then checked for growth, sealed and stored at room temperature in a closet. At set intervals, ranging up to one year, all three strains were checked by opening some test tubes from each series, and using the material to make smear preparations and starting control cultures on McCoy's medium.

It was found that Bact. tularensis survived in liquid egg yolk as long as the experiment lasted, and on the other hand, that under the same conditions on McCoy's medium, it died within three months. Later findings convinced the author that Bact. tularensis remains viable in the liquid medium for well over a year.

It should be possible to take advantage of this special characteristic of the liquid egg yolk medium in many different studies on the tularemia agent.

For example, it could be used to preserve type culture strains ("museum" strains) of *Pasteurella tularensis*. Cultures kept in it would have to be transplanted less frequently than if kept in McCoy's medium, without risk of impairing their viability.

Virulence of Bact. tularensis in liquid egg yolk medium. It is a well known fact that protracted growth in artificial culture media reduces the virulence of many pathogenic microorganisms and that virulence can be restored only through animal passages. This is absolutely true of *Bact. tularensis*, e.g., Foshay grew two highly virulent strains in McCoy's medium and after keeping them at 10° for 10 weeks obtained a strain which was avirulent in mice. At the same time, the control strain retained its full virulence. In his studies on vaccination with live, attenuated cultures, Kudo attenuated his strains by growing them in an egg yolk serum medium at room temperature for 80 days. According to Francis, cultures grown in egg yolk retain their virulence longer than those grown in agar. According to Khatenever, strains kept for long periods in McCoy's medium, gradually lose their virulence. Kudo's studies on the properties of *Pasteurella tularensis* strains kept in different media prove that strains grown in McCoy's egg yolk medium and in egg yolk serum were virulent in white mice and guinea pigs even after 60 subcultures, while the virulence of a culture grown in cysteine-agar was drastically depressed already after 30 subcultures. According to his experience, the virulence of cultures stored at room temperature (after 50 subcultures) was best preserved in McCoy's coagulated egg yolk medium, being only slightly reduced after three months of storage. In egg yolk serum, the virulence was preserved for two months, while in cysteine-agar media, the strains were avirulent in mice already after 2 months. Larson proposes to preserve the virulence of *Pasteurella tularensis* strains by growing them in the chorio-allantoic membrane of developing chick embryos.

There is a work by Deinsen on the virulence of bacteria in egg yolk media. According to this author's observations, human strains of the tubercle bacillus, if cultured in Besredka's medium, retain their full initial virulence for 4 months. Bovine-type strains remain virulent much longer, namely, up to ten and even twelve months.

Murontsev mentions in his work on semi-liquid culture media that very many pathogenic bacteria are characterized by the fact that their cultures remain highly virulent for long periods of time. He explains the heightened virulence of strains grown in semi-liquid media by this - that such media offer bacterial growth conditions much more like those encountered in the living organism, and that growth in them is like a passage replacing animal passages.

The virulence of *Bact. tularensis* grown for long periods in liquid egg yolk was checked by the author on a series of test tubes with liquid egg yolk medium which she had inoculated with strains No. 9 and No. 14, using single inoculation doses of 1 million bacterial bodies. Both of these strains had been previously titered on white mice. After having been incubated for two days in the thermostat, the test tubes were sealed and set aside at room temperature for long storage tests. At set times, some of the test tubes were opened and their contents transferred to McCoy's medium. The McCoy cultures were emulsified and used for inoculating white mice. The results obtained with strain No. 9 are presented in Table 1.

The data presented in Table 1 prove that during the first six months the virulence of the strain not only failed to drop, but rose slightly even over its initial value. This seeming paradox, i.e. increased virulence after a long period of growth in an artificial culture medium, was also mentioned in his days by Deinsen, whose experience indicated that the virulence of some of the human types of the tubercle bacillus is slightly increased after this bacillus has been grown for two months on Besredka's medium.

The author found that the initial virulence of the tularemia agent remained practically unchanged after one year in the liquid egg yolk medium (without reseeded).

Agglutinability of *Bact. tularensis* in liquid egg yolk medium.
In tularemia, the agglutination test is extremely important because of its high specificity. It serves both as a diagnostic tool, and as a means of culture identification. Hence, it was important to establish the extent to which the agglutinability of the cultures remains preserved, when these cultures are grown over long periods of time in the liquid egg yolk medium. Consequently, special experiments were performed demonstrating that there is no change in the agglutinability

of *Pasteurella tularensis* strains even after a protracted stay in this medium. Their agglutination titers remain absolutely the same and the agglutination reaction produced is as definite as the one produced by strains kept in McCoy's medium.

The findings concerning the growth properties of *Pasteurella tularensis* strains in the liquid egg yolk medium prove, as demonstrated in the present paper, that this medium is superior to all the other currently used media, because of the following characteristics: the minimum inoculation dose sufficient to produce active bacterial growth is very low (5 million bacterial units); proliferation in it is so active that even after one day of growth, one loop of the material subcultured in McCoy's medium, produces a solid layer of bacterial growth, and finally because it is a medium in which the tularemia agent retains its viability, virulence and agglutinability.

The definite superiority of this medium over other culture media is explained by the fact that it contains all the nutritive and growth-promoting substances required by the tularemia agent, and that due to its physiochemical characteristics, it offers optimum growth conditions to *Pasteurella tularensis*.

Muromtsev explains the excellent qualities of the semi-liquid culture media he employs by the fact that their physiochemical properties make them very similar to the natural juices of the living organism, and he stresses the importance of such a factor as the viscosity of the medium.

According to the published data, bacterial growth is stimulated when the viscosity of the medium is increased, but only up to a certain level. When the viscosity increase exceeds that level, it has the opposite effect (Koser, Chinn and Sander, Golubova, etc.).

It is possible that in the case of highly viscous culture media, the growth inhibiting factor is the slow bacterial diffusion rate in the medium. Hence when selecting a culture medium for a given micro-organism, one should consider its viscosity so as to ensure optimum growth conditions.

The excellent properties of the liquid egg yolk medium are demonstrated, moreover, by the morphology of *Bact. tularensis* in this medium. The forms cultured in it resemble those usually encountered in contact preparations from the organs and tissues of animals infected

with tularemia. For example, organ preparations from animals dead of the disease, show the pathogenic agent in the form of little rods. Similarly, in the liquid egg yolk medium, *Bact. tularensis*, assumes also rod-like though larger forms.

Conclusions

1. In the liquid egg yolk culture medium, *Pasteurella tularensis* retains its basic characteristics for a long time. The author could establish in her studies that *Pasteurella tularensis* strains retain their full viability, virulence and agglutinability characteristics for one year, and longer.

2. The liquid egg yolk medium is an ideal culture for *Pasteurella tularensis*, because due to its physiochemical properties and the fact that it contains all the indispensable nutritive ingredients it offers this microorganism optimum growing conditions.

Table 1.
Virulence of *Pasteurella tularensis* after long growth in liquid egg yolk medium without reseeded

Experimental animals	Infectious dose	Length of storage periods				
		Initial virulence	1.5 months	3 months	6 months	12 months
White mice	1 mill. b b	2	2	2	2	2
	100,000 b.b.	4	4	3	4	5
	10,000 b.b.	4	4	4	4	5
	1,000 b.b.	5	5	4	4	5
	100 b.b.	7	4	4	4	5
	10 b.b.	8	7	7	7	7

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Summary.

Studies on the viability, virulence and agglutinability of Pasteurella strains kept over long periods of time in liquid egg yolk medium have demonstrated that in cultures these characteristics of the bacterium remain unaltered for a long time.

April 13, 1961
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Translated at the
National Institutes of Health
Bethesda, Maryland