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From the Russian for
Dr. John F. Bell

Trudy Rostovskogo na-Donu
[illegible]
protivochumnogo [illegible], VI
[Tularemia], 71-82, 1947.

Zhidkaya zheltochnaya sreda dlya vyrashchivaniya kul'tur tulyaremii

Soobshcheniye II*. Kharakter rosta tulyaremiynogo mikroba v zhidkoy zheltochnoy srede

A liquid egg yolk culture medium for growing Pasteurella tularensis

Report II*. Growth characteristics of Pateurella tularensis in liquid egg yolk medium

by

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*Report I was published in Vol. IV of the Transactions of the Institute.

After initial studies on the selection of the optimum egg yolk concentration in the medium, the minimum seeding dose, and some animal experiments, the author extended her studies to an investigation of the properties of the culture medium itself and the growth characteristics of the tularemia agent in this medium.

Determining how long the medium can serve. In everyday work, the question how long a medium can be used becomes very important. For example, the principal disadvantage of McCoy's medium is that it must be freshly prepared. Even one month from the date of preparation it may no longer be serviceable as a culture medium. Under field conditions, when the proper facilities are lacking, the necessity of preparing fresh batches of the medium presents very serious difficulties. It was definitely important, therefore, to find out whether a liquid egg yolk medium having good storage characteristics could be developed. Therefore, a special experiment was set up to determine how long the liquid egg yolk medium in question would remain effective. Thus, the freshly prepared medium was poured into flasks and placed in a closet at room temperature for longer storage. Before being filled with the medium, the flasks were checked to ensure sterility and, once filled, closed with rubber caps. During the first six months following the date of preparation, the flasks were opened once a month and the medium was distributed into test tubes. The latter were seeded with various doses of the two tularemia strains selected for the purpose.

The experimental findings demonstrated that the culture medium retained its properties during the whole test period. It was found that the tularemia agent grew just as well in a medium prepared six months before, as in freshly prepared medium. Consequently, the liquid egg yolk medium can be prepared in advance and it can be taken for granted that the storage dates determined do not represent limit values. Inasmuch as it has been established that long storage does not affect the properties of this particular medium, it can be definitely stated that it can be prepared in advance and shipped to central supply points for subsequent distribution to peripheral and secondary laboratories and epidemiological stations.

The liquid egg yolk medium was prepared in advance and tested by the author on her field trips to epizootic tularemia foci and areas where the disease had broken out. The liquid medium was transported to these points in flasks with rubber stoppers, sealed with Mendeleyev sealer, then transferred, when required for local use, to sterile test tubes. It proved invariably effective for culturing bacteria isolated from animal sources.

Proliferation and "M" concentration of the tularemia agent in liquid egg yolk medium. It has been demonstrated by the works of Bailey, Claypon, Chesney, Penfold, Buchanan, and others that bacterial

proliferation in liquid culture media proceeds strictly according to certain laws of growth. The fact that the bacterial population proliferates according to certain fast rules can be easily verified by making a periodic count of the bacterial cells in a culture. A graph, where the number of bacteria is recorded on the ordinate and culture time on the abscissa, yields a typical growth curve: Immediately after seeding there is a short period when there is no proliferation. Then the number of bacteria begins to increase; first slowly then rapidly, reaching at a given moment a level at which it remains constant for a time. This is followed by a period when the total number of viable microorganisms declines.

The works of Sherman, Cameron, Dubos, etc., have shown that the growth of the bacterial population may be drastically affected by such factors as temperature differences between mother and daughter cultures, the age of the mother culture as well as the size of the seeding dose.

As a result of his studies on the entero-typhoidal group of bacteria, Bailey reached the conclusion that at the peak of the stationary phase, the number of bacteria per unit volume of the medium is at its peak, and that this maximum is always an absolute constant for each bacterial type. It is Bailey who introduced to the microbiological vocabulary the term "M" concentration, i.e., the concept that each bacterial strain has a characteristic maximum population density.

Different bacterial types differ by their "M" concentrations even under absolutely identical conditions.

The "M" concentration of each type in a given culture medium is an absolute constant. When the seeding dose exceeds the "M" concentration of the strain, the surplus bacteria die off and the characteristic "M" concentration is soon re-established.

Since bacterial growth rate and "M" concentration are factors which can serve as good indicators of the quality of the culture medium and can determine its application in a number of situations arising in the course of studies on a given bacterial type, the author set up experiments to that effect with the tularemia agent in liquid egg yolk culture medium.

It has been found that for studies on bacterial growth in liquid media the most appropriate are the direct bacterial cell count methods, i.e., either a count of the colonies growing on the culture plate, or a count of the viable bacteria in limit dilutions corresponding to the initial proliferation values.

In the present work, the author had used the second of these methods. She inoculated with material from two-day tularemia cultures 100 cc of liquid egg yolk medium, the inoculation dose being 100,000 bacterial cells per 1 cc of the medium. The batch was carefully stirred and titered into a series of test tubes which had been carefully sterilized before being filled with various dilutions (ten-fold dilutions) of the culture medium. The test tubes were seeded using a different pipette each dilution. The pipettes employed in this operation were graduated and carefully calibrated. Both mother and daughter cultures were grown at 37°C. At set times, samples were taken from the mother cultures and titered, as before, into a series of test tubes. In order to nullify the effect of temperature differences, the test tubes with the culture medium were heated in a thermostat before seeding to a temperature of 37°C. The growth of the tularemia agent in this medium was studied in two strains: No. 9 and No. 13. The mother cultures were kept under observation for 80 days.

Evaluation of the results consisted of a microscopic examination of the culture smears, and tests in which material from the test tubes was grown in the thermostat in McCoy's medium for five days.

The experimental data obtained prove that the initial stationary phase in this case is very short, since already two hours after seeding one can note the first signs of proliferation, and during the next few hours the number of bacteria rises to 100 million/cc of the medium. Within 36 hours, bacterial concentration in this experiment reached its "M" value, the count being 1 billion bacterial cells/cc of the medium.

For graph see page 74.

The numbers along the abscissa indicate the age of the culture, in days. Those along the ordinate give the bacterial count.

The curve in this graph shows that the stationary phase of peak proliferation with the Bact. tularense is very long in the liquid egg

yolk medium and that at 37°, the bacterial count does not begin to drop until the 9th day. The count continued to decline for many days so that it was still dropping on the 60th day, which marked the end of the observation period. The general growth characteristics and high "M" concentration of Pasteurella tularensis in the liquid egg yolk medium prove that the latter is an excellent culture medium for this micro-organism.

The morphology of Pasteurella tularensis in the liquid egg yolk medium. The morphology of the tularemia agent in this medium presents many interesting features.

Francis, Park, Calli-Valerio, Somov, Khatenever, Sinay, Dvizhkov, Levchenko, and Volferts were all able to demonstrate in their studies that the tularemia agent is extremely polymorphous and that it may occur either in coccal form or in the form of short rods. They proved further that the coccal forms predominate in older laboratory strains grown for long periods of time in artificial nutrient media, and that a predominance of rod-like forms characterizes the newly isolated strains.

Khatenever, Sinay, Francis, Dressel, and others report that cultures grown in liquid media contain curved, thread-like, frequently granular forms the coloration of which is often bi-polar.

O'Hara, Kudo and Kubayashi mention the unusual polymorphism of Pasteurella tularensis and correlate this fact with the virulence of the strains. The findings of these authors indicated that the more virulent a culture, the more diversified the morphology of its forms, and the less uniform its color. When virulence decreases, the bacteria assume a coccal form.

On the strength of studies performed on 43 strains varying as to virulence and isolated at different dates, Hesselbrock and Foshay conclude that the tularemia agent displays a great diversity of forms, the most typical of which are those common to all Pasteurella tularensis strains: large and small coccal forms, flat or cylindrical rod-like forms, thread-like and very large spherical forms, as well as the so-called filtrable reproductive forms so minute that they appear as mere pin-pricks. The authors think that all these peculiar and unusual entities are not involutional, but that they represent actual and typical forms of this pathological agent, representing various periods of its growth cycle.

In liquid egg yolk medium smears, Pasteurella tularensis appears in general much larger then in solid culture media, and its form is definitely bacilliform. When carefully examined, the liquid medium smears show (especially during the first 15 - 24 hours of growth) many bacteria arranged in pairs and interconnected by tenuous filaments. Khatenever and O'Hara mention these thread-like shadows which seem to link the separate bacteria, and O'Hara regards them as a characteristic of the tularemia agent. The presence of these filaments indicates a very active process of proliferation. Occasionally, the bacteria in the smears are arranged in fours and fives, forming short chains. As the density of the bacterial population rises, these chains become progressively shorter, their links gradually assume the form of cocci which form, by and by, aggregates and "clumps."

In her own studies, the author could observe many unusual forms, e.g., vibrioid forms, clavated forms resembling dumbbells, as well as some extremely long and coiled forms. She found that in general these unusual forms stain very unevenly and that they often contain one or two deeply stained granules.

During the early proliferative stages, i.e., at those times when proliferation is very active, one often observes relatively long chains of bacteria which remain linked for a time by tenuous filaments. This coincides with the findings of Imshenetskiy, who called attention to the elongated Proteus vulgaris forms found around the periferies of the colonies of this microorganism. When they are stained one can see that they contain granules. It was thought at first that these granules represented nuclei, but subsequent cytological studies proved this concept to be erroneous. It was found that these granules belong to individual bacteria which are not separated, still lacking dividing walls. Later there is an equalization of the rates of growth and proliferation. The long Proteus rods separate to form individual bacterial bodies. The same occurs, apparently with Bact. tularense in the course of its growth cycle: during the period of active growth some of the bacterial cells cannot detach themselves from the mother cell, and present the appearance of long filaments containing various inclusions. Hesselbrock and Foshay on observing such forms thought that they consisted of several, still unseparated bacteria. When material from cultures containing such peculiar forms was seeded in McCoy's medium, the bacteria grown were invariably true to their morphological type.

When liquid egg yolk smears are stained, it should be remembered that in the preparations the culture medium is stained too, and that it forms the background for the stained bacteria. Hence, contrast staining techniques are recommended. In Giemsa stained preparations the bacteria are stained violet, the background pale pink. Protracted staining (2-3 hours) in aqueous methylene blue solutions gives good results: the bacteria are stained deep blue, the background light blue. Fuchsin-bluing, used as indicated by Muromtsev, produces satisfactory results with semi-liquid culture medium preparations. This is a very rapid contrast-staining method.

With these smears it is best to employ wet fixation methods. The author, in her work, used the Nikiforov mixture and staining times of 10-15 minutes. The conventional flame-fixation method gives poor results, inasmuch as it often causes the smears to wrinkle or even wash off in water.

Studies on the motility of Pasteurella tularensis. The motility of the tularemia agent has been studied by many, but remains a moot question.

The findings of Kudo, O'Hara and Kubayashi indicate that Pasteurella tularensis can move actively. These authors are of the opinion, furthermore, that there is a certain correlation between the motility of a strain and its virulence. They make use of special staining methods to demonstrate that bacteria cultured from such strains were provided with flagella.

Soviet as well as American scientists reject the assumption that Pasteurella tularensis is a mobile microorganism, since none of them have succeeded in observing it in motion. Vereninova, for example, studied twenty different strains of Pasteurella tularensis in hanging-drop cultures, but could never observe any active movement.

Hesselbrock and Foshay discuss this matter exhaustively in their latest work. Using certain highly complicated supravital staining methods, they were able to ascertain that the bacteria were provided with delicate filaments which linked them into groups. In fixed preparations these filaments were often broken and looked like flagella, which apparently must have misled O'Hara and his coworkers into thinking they were true flagella. These "flagellate" forms, however, were perfectly motionless.

After having studied numerous preparations (actually thousands of preparations) the authors became convinced that Pasteurella tularensis is motionless. They feel that the filaments one observes in the smears are not real flagella, but a special feature characterizing one of the growth stages of this particular microorganism.

The writer of the present article, being anxious to investigate the question of the motility of the tularemia agent for herself, seeded liquid egg yolk cultures with ten strains: Nos. 9, 13, 14, 10, 5, 23, 28, BK1, and BK4. The mobility of the microorganisms was studied throughout by the hanging-drop method after the cultures had been incubated for twenty-four hours in the thermostate. The cultures for this study were grown at different temperatures, thus, at 37°, 28° and 20°. They were cultured at different temperatures for this reason that many microorganisms are more active at lower temperatures. For example, B. pseudotuberculosis Pf. is motionless when grown at 37°, but quite active when cultured at 18-20°.

Although the author grew and studied in liquid egg yolk medium ten different strains of the tularemia agent(strains which differed as to their virulence),not once was she able to detect any movement. In some highly virulent strains (for example, in Nos. 31, 14 and KM1), the brownian movement was quite distinct; it was so marked in some cultures grown at lower temperatures that it almost gave the appearance of slow motion.

Though the experiments were repeated many times, the results obtained were always the same.

Pasteurella tularensis grown in liquid egg yolk medium at different temperatures. Contrary to most bacteria which can grow within a temperature range of 3 or 4° to 45°, the tularemia agent proves in artificial culture media highly sensitive to temperature changes.

In McCoy's medium, even when grown at the optimum temperature of 37°, Pasteurella tularensis begins to show the first signs of proliferation only after 48 hours. At lower temperatures it generally fails to grow. Hence, the temperature has to be closely watched, particularly when the bacterium is to be grown from freshly isolated animal material. This is a great complication, since under certain field conditions and with certain types of equipment, it is practically impossible to obtain the optimum temperature level.

The author set up special experiments in order to study the possibilities of culturing Bact. tularense at lower temperatures. Nine different strains were seeded and grown at 37°, 28° and 20° (Table 1). In McCoy's medium, which was used in these tests for the controls, there was no proliferation at 37° for the first 48 hours. At 28° only a few strains produced a weak growth by the 5th to 7th day.

It must be mentioned, however, that in the liquid egg yolk medium, at temperatures less than optimum, bacterial proliferation is much slower. For example, while bacterial concentration in smears from cultures grown at 37° is comperatively high already after 48 hours, in smears from cultures grown at 28° it is lower, and in smears from cultures grown at 20°, there are only a few scattered bacteria.

Table 1.
Bact. tularense cultured at different temperatures.
(seeding dose 10,000 bacterial organisms)

Strain	Incubation temperature					
	37°		28°		20°	
	Liquid egg yolk med.	McCoy's medium	Liquid egg yolk med.	McCoy's medium	Liquid egg yolk med.	McCoy's medium
No. 9	+++	+++	++	-	+	-
No. 13	+++	+++	++	-	+	-
No. 31	+++	+++	++	-	+	-
No. 14	+++	+++	++	-	+	-
SKI	+++	+++	++	-	-	-
No. 10	+++	+++	++	-	+	-
No. 25	+++	+++	++	-	+	-
No. 23	+++	+++	++	-	+	-
No. 28	+++	+++	++	-	+	-

Symbols: +++ = very active proliferation
++ = active proliferation
+ = slight proliferation
- = no proliferation.

Remark: proliferation data recorded for a period of two days (= 48 hrs).

Numerous experiments with various seeding doses have demonstrated that Pasteurella tularensis is less sensitive to temperature variations in the liquid egg yolk medium.

Bact. tularense will grow in this medium even at 28°C.

Conclusions.

1. As proven by the bacterial growth rate and high "M" concentration, liquid egg yolk is an excellent culture medium for Pasteurella tularensis.

2. In the liquid egg yolk medium, the Bact. tularense has the same morphology as in impression preparations from animal organs. This proves that for growing Pasteurella tularensis the liquid egg yolk medium is better than other media, because its composition is very similar to that of the medium in which this microorganism occurs in nature.

3. The liquid egg yolk medium does not deteriorate in storage. According to the author's own experience, it can be effectively used for upwards of six months.

4. Bact. tularense is less sensitive to temperature variations in the liquid egg yolk medium, and can be grown in the latter even at lower temperatures (according to the author's experience, even at 28°).

5. The liquid egg yolk culture medium proposed will make it possible to initiate comprehensive studies on such imperfectly clarified subjects as the pathogenicity, morphology, motility, enzymatic properties and other characteristics of the tularemia agent.

Summary.

Studies on the growth characteristics and morphology of Bact. tularense in liquid egg yolk medium as well as the keeping properties of the latter ~~have~~ demonstrated that this medium rapidly produce Bact. tularense cultures from minimum seeding doses.

The growth characteristics, morphology and "M" concentration of Bact. tularense in the medium in question prove that the latter is superior to other culture media used for the purpose.

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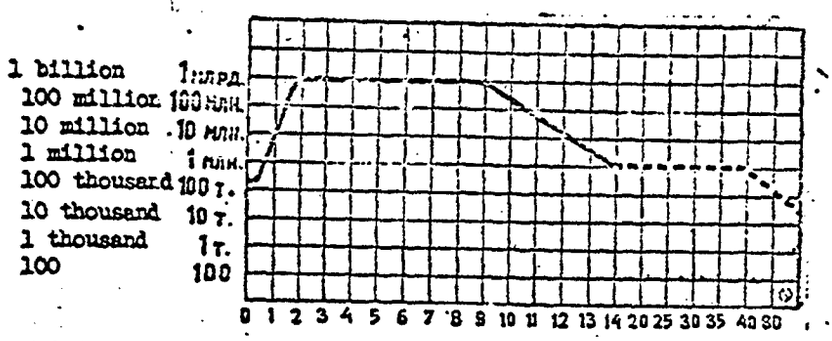
Attachment to No. 4-11-61 [1 of 1]

Proliferation of tularemia strain No. 13 in liquid egg yolk medium at 37°C. Initial seeding dose: 100,000 bacterial bodies.



На оси абсцисс отложено время выращивания в сутках. На оси ординат отложено количество микробных клеток.

Как видно из приведенной графической кривой, максимальная стационарная фаза палочек туляремии в жидкой желточной среде очень продолжительна и, только начиная с 9-го дня при т-ре 37°, начинается медленное уменьшение общего количества микробов. Отмирание микробов затягивается на продолжительный срок и не заканчивается еще к 80-му дню (срок наблюдения). Общий ход размножения туляремийных микробов в жидкой желточной среде и высокая «М»-концентрация указывают на оптимальность этой среды для выращивания возбудителя туляремии.

Морфология микробов туляремии в жидкой желточной среде. Морфология туляремийного микроба в жидкой желточной среде представляет ряд интересных особенностей.

Работами Френсиса, Парка, Галли-Валерио, Сомова, Хатеневера, Синая, Движкова, Левченко, Вольферц показано, что палочки туляремии — чрезвычайно полиморфный микроб, встречающийся в виде кокков и коротеньких палочек. При этом, для старых лабораторных штаммов, длительно хранившихся на искусственных питательных средах, характерным является преобладание кокковых форм, в то время как у свежевыделенных штаммов превалируют палочковидные формы.