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THE PREPARATION OF BRUCELLA PHAGE AND THE DETERMINATION OF
SOME OF ITS PROPERTIES

(Uzbek Scientific-Research Veterinary Institute)

- USSR -

[Following is a translation of an article by Candidate of the Veterinary Sciences E. G. Mamatsashvili. The article appeared in the Russian-language book Sbornik Trudov Mezhhinstitutskoj Nauchnoj Konferentsii Bakteriofagiya (Symposium of the Work of the Inter-Institute Research Conference on Bacteriophages) Tiflis, Vol. 5, 1955, pp 327-332.]

The growth of brucella cultures on dense nutrient media (in test tubes on slanted agar, in Petri dishes on agar) is characterized by the fact that colonies are at first delicate, transparent, convex, round with correctly contoured periphery, homogenous or with barely perceptible granularity. In time (with inocula from the infected organism of man or animals, as well as from pathological material - after 5-10 days, and sometimes even after 2-4 weeks; during re-inoculations - after 48-72 hours) these colonies become turbid and take on a more distinct character: colonies disperse into individual isolated small grains and gradually decrease in size. The space between colonies, rather, between grains of individual colonies, gradually increases. Where not long before there was a continuous growth of culture there arise quite smooth matty surfaces. The culture of brucella is gradually recedes with time.

The observed picture, especially when working with cultures of museum strains, suggests the idea that brucella cultures contain bacteriophage.

Reports of Soviet investigators (Ye. O. Nemsadze, F. Ye. Sergiyenko, M. S. Drozhevkina), as well as of foreign authors (M. L. Pickett, S. H. Williams), positively deciding the questions of the existence of brucella bacteriophage, acquired for us a very real significance. However under these circumstances it was considered that brucella, as a biological unit within a vast world of microorganisms, is isolated into a special independent group with these types. Also considered was the well known wide mozaic of the antigen structure of these microbes. It was therefore proposed that the preparation of brucella bacteriophage and the character of its several properties will be unique.

The success of the work, as it often happens, depended on the selection of a method of investigation. In our view the question of developing a method for the isolation of brucella bacteriophage was successfully resolved by M. S. Drozhevkina. Using the method developed by this author, in August 1954 we started the isolation of brucella bacteriophage and the study of its several properties.

Of the 28 local strains of *Br. melitensis* available in the laboratory of YzNIVI (Uzbekskiy nauchno-issledovatel'skiy veterinarnyy institut - Uzbek Scientific-Research Veterinary Institute) we succeeded in isolating brucella bacteriophage from 13 strains. 9 of these strains had a term of isolation of 20 to 32 months, and 4 were freshly isolated with a term of isolation of no more than 4 months.

Of the total number of 21 local strains of *Br. bovis* available in the laboratory, we succeeded in isolating brucella bacteriophage from only two strains. The latter had a term of separation of 8 months.

Local strains of *Br. suis* were not available, accordingly it was necessary to work with strains isolated by other authors in 1932 and 1935 in Moscow, Novosibirsk and Canada. After repeated attempts we also succeeded in isolating brucella bacteriophage from all three available strains of *Br. suis*.

At the end of 1953 the term of suitability of a live brucellosis vaccine from strain No. 68, series 6 and 7 ran out. In deciding the question of the suitability of such a vaccine as a customary material for the immunization of agricultural animals against brucellosis it was necessary to make inocula from flasks of this vaccine in dense nutrient media. The character of growth of these inocula was such that the presence in them of brucella bacteriophage in them could be unmistakably supposed. Further, brucella bacteriophage was isolated from these cultures.

In the following stage of the work brucella bacteriophage was isolated from this same vaccine, directly with the first inoculum. In this the vaccine series No. 20 was used, its term of suitability having not run out. Brucella phage was also separated from flasks of this bioprep.

Brucellosis vaccine from strain No. 68, as is well known, is prepared from weakly virulent strain No. 68 and is a fine grayish-white suspension of microbes of brucella and aluminum hydroxide in physiological solution. After the demonstration of brucella phage in this liquid vaccine, at once the question arose - do dry live brucellosis vaccines contain bacteriophage? Three forms of vaccine were used to decide this question: brucellosis vaccine (strain No. 19), prepared by the Kashintsevskaya Biofabrika (Biological Plant, series No. 61, brucellosis dry live vaccine NIIEG (Nauchno-issledovatel'skiy institut epidemiologii i gigieny - Scientific-Research Institute of Epidemiology and Hygiene) series 115, and finally, brucellosis dry vaccine IIm AMN SSSR (Institut eksperimental'noy meditsiny, Akademiya meditsinskikh nauk SSR - Institute of Experimental Medicine, Academy of Medical Sciences USSR) series 363. We also succeeded in isolating brucella phage from all three forms of vaccine.

Further, the brucella phage was separated from yet another brucellosis vaccine - namely a vaccine of strain No. 12 - UznIVI. This vaccine is, for the time being, in a stage of experimental investigation.

The establishment of the presence of brucella phage in brucellosis vaccines dictated the establishment of this phage in the organisms of animals immunized by these vaccines. In this direction as yet only one fragment of work has been conducted, specifically: in one large cattle farm, insecure with respect to brucellosis, in which preventive measures for this disease is being realized with the immunization of animals with brucellosis vaccine from strain No. 68, brucella phage was successfully separated from 14 fecal probes, 7 urine probes and 7 milk probes after 11 months following vaccination. It is characteristic that these probes of feces, urine and milk belonged to those cows which at the moment of taking the material showed a high agglutination titre (in most cases - 1:400, rarely - greater than 1:200), and 50% of these animals had positive RSK indications.

The presence of brucella phage was established by a biological means during which certain properties of the phage were determined at the same time. The presence of the phage is acknowledged if the liquid, passed through Chamberland filters L₃ and L₅, cause lysis of brucella on plots of an 18-hour brucella culture in Petri dishes, on MPPA, with the copious growth of those microbes in the control portion of the dish, but without the reaction of the filtered liquid.

After establishing specificity, the titres of breeds of the isolated phage are Appel'man modified in Martin's broth after Appel'man, during which the question of the activity of the brucella phage on brucella in a liquid nutrient medium is decided at the same time.

For the confirmation of the presence of each breed of the isolated brucella phage by testing it on a solid nutrient medium, as well as for titration of each separate breed of phage by the method of Appel'man, all strains of all three types of brucella available in the laboratory were used. Hence it was ascertained that of the

number of 53 breeds of brucella phage, 13 breeds were sensitive to one strain of brucella, 5 breeds to two strains, 7 breeds to three, four, five and six strains, 4 breeds to seven strains, and finally, one breed to eight, nine, ten, and twelve strains.

Of the total number of isolated breeds of brucella phage, 18 were effective for one type of brucella, 25 breeds - for two types, and 9 breeds to all three brucella types. However it should be considered that among the breeds which were effective for one type of brucella, they were in most cases sensitive to Br. melitensis (15 breeds), and only rarely to Br. bovis (1 breed) and Br. suis (2 breeds). We have an almost analogous picture even with respect to those breeds of phages which had a simultaneous effect on two types of brucella, namely: lysis of brucella types melitensis and bovis -- 22 breeds, of brucella types melitensis and bovis - 2 breeds, and finally, of brucella types bovis and suis - 1 breed.

The question of type specificity of breeds of brucella phage merits special attention. Its thorough study and solution will have theoretical interest and great practical value, for possibly a new method will appear for improving the presently existing classification of brucella groups, as well as methods for their differentiation. Work on this idea has just begun. However, in this, consideration of the fact that other factors also have a definite affect on the sensitivity of different types of brucella to bacteriophage, must not be neglected. It should especially be noted that when an isolated breed of brucella phage displayed the capability of simultaneous activity on several strains of brucella of three or two types, these strains had always been freshly isolated, and consequently, the brucella phage displays its greatest activity against freshly isolated brucella strains. Three strains of Br. suis were comparatively phage-resistant. They were subjected to lysis to a lesser degree, and possibly this was so not because they were Br. suis, but because these strains were isolated long ago. This question requires further clarification.

Titres of just isolated breeds of brucella phage are relatively small; according to the Appel'man method they do not exceed 10^{-2} , 10^{-3} . In some breeds of phage titres were so small they could not be titrated by the Appel'man method. However it was possible to raise their titre after repeated passage. Thus, for example, two breeds of the eighth generation showed an Appel'man titre of 10^{-9} . In passage this curious fact developed: often breeds of brucella phage after passage acquired a sensitivity to those brucella strains with respect to which they showed no activity in their freshly isolated form.

The activity of one and the same breed of brucella phage on brucella in dense nutrient medium at the same time that on brucella of the same strain in liquid nutrient medium no activity is displayed merits special attention and requires further study.

Along with biological study of isolated brucella phage, an attempt was made to examine it in the electron microscope and photograph it.

The electron microscope in the Physical-Technical Institute of the Academy of Sciences of the Uzbek SSR was used. Considering that we, as well as the scientific co-worker of the above institute, N. V. Kardub, who managed and directly operated the electron microscope, had no experience in working with phages in general, and with brucella phage, in particular, we therefore began by first examining and photographing sterile Martin's broth, then dysentery phage prepared at the Tbilisi Scientific-Research Institute of Vaccines and Sera, series 396, and finally, brucella phage, separated from a vaccine of strain No. 68, series 6.

The presence of brucella phage was confirmed by the electron microscope.

The work conducted allows us to make the following preliminary conclusions:

1. The character of growth of brucella on dense nutrient media provided a basis to suppose that bacteriophage exists in brucella cultures.
2. Starting in the month of August 1954, we succeeded in isolating 53 breeds of brucella phage from museum brucella cultures of all three types of brucella, from liquid and dry brucellosis vaccines, and from the feces, urine and milk of animals vaccinated.
3. Titres of freshly isolated breeds of brucella phage as a rule are weak, however, after repeated passage we succeeded in bringing the titres of two breeds of this phage up to 10^{-9} for the Appel' man method of titration.
4. The isolated breeds of brucella phage do not fit into the presently accepted classification of brucella groups with respect to their sensitivity to the various brucella types.
5. Isolated breeds of brucella phage display far greater activity against freshly isolated strains than they do against cultures of museum strains.
6. One and the same breed of brucella phage, effective on brucella in dense nutrient medium, does not have an effect on brucella of the same strain in liquid nutrient medium.
7. The presence of brucella phage is confirmed not only by biological means, but also by means of electron microscope examination and photography.