Notes

Cloning of Leptospires by Micromanipulator

by P. BAKOSS [1]

Monocell leptospiral cultures are a fundamental requirement in genetic research and are not infrequently helpful in practice, for instance, for separating homogeneous cultures from mixed ones. Three methods of cloning of leptospiral cultures are mentioned in the literature: by micromanipulator (Bessemans & Derom, 1948), by dilution (Babudieri, 1966) and by cultivation on solid media (Larson et al., 1959; Cox, 1966).

In this paper our experience with the micromanipulator technique is described. The method was used for experiments in which the antigenic changes of leptospiral strains cultivated in the presence of homologous or related heterologous immune rabbit sera were investigated.

Materials and methods

The investigations were performed on leptospiral strains originating from the strain Veldrat Batavia 46 (*javanica* serotype). A smaller group of clones was obtained from strains belonging to the Bataviae and Australis serogroups.

A micromanipulator made by Carl Zeiss, Jena (Gleitmikromanipulator) with only one operation stand was used. A thin polyethylene tube with a diameter of 1.5 mm was fastened to the holding-clip. A sterile glass micropipette filled with Korthof's medium was inserted into one end of this tube and a syringe was connected to the other end in order to obtain a simple pneumatic system. A loop of a leptospiral culture, diluted so that one leptospire was detectable on 1 or 2 dark fields was spread out in a thin layer on a sterile slide on the microscope table. (A wet chamber was not used as its thickness does not transmit a sufficient quantity of light in dark-field observation.) One leptospire was aspirated by syringe into the micropipette and transferred into the cultivation medium. A single micropipette was used for each isolation. The whole operation was performed under sterile conditions.

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The well-grown strains were compared in the agglutination test with the original strain. Strains showing deviating antigenic properties were later, after repeated cloning, stored in the collection of strains.

Altogether 99 cultures of leptospiral strains were cloned; 5–15 tubes were inoculated out of each culture. The total number of cultivated tubes was 766. In the first group of clones (31 cultures) only the numbers of positive, negative and contaminated tubes were determined. With the remaining two-thirds of the clones (2nd group) the dynamics of growth was in addition microscopically investigated for 5 weeks by checking the cultures at 7-day intervals. Of the leptospiral cultures used for cloning in the 2nd group, 45 were 6–12 days old, 22 were 13–19 days old and 1 was 4 days old.

Results

The results of these investigations are summarized in the table. Out of the 766 inoculated tubes, leptospires were found growing in 55.3%; 38.6% of tubes were negative and 6.1% were contaminated. The results of the 1st and the 2nd groups were quite similar. The smaller number of grown clones in the 1st group is obviously due to the higher percentage of contaminants.

After 1 week 17 tubes (5.6%) out of the total number of 302 positive tubes in the 2nd group were found to contain leptospires. The highest number of positive tubes—201 (66.6%)—was found after 2 weeks. A further 64 (21.2%) were found to be positive after 3 weeks, a further 12 (4.0%) after 4 weeks, and a further 8 (2.6%) after 5 weeks.

Concerning the intensity of growth, about two-thirds of the clones took 21–38 days to grow into rich cultures suitable for the agglutination test. Approximately one-third of the clones grew very poorly, so that the strains had to be transferred into new media, but in spite of this a small number of strains was lost.

During our investigation we noticed that it was sometimes very difficult to clone a new (antigenically

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Clones	Number of cultures cloned	Growth of leptospires in tubes			
		Positive	Negative	Contaminated	Total
1st group	31	121 (46.7%)	107 (41.3%)	31 (12.0%)	259
2nd group	68	302 (59.6%)	189 (37.3%)	16 (3.1%)	507
1st + 2nd groups	99	423 (55.3%)	296 (38.6%)	47 (6.1%)	766

changed) culture that was otherwise growing well. In some cases the isolation of clones from such cultures was successful only after 3 or 4 repeated clonings. This did not depend on the age of the culture and was apparently due to slight changes in the composition of the cultivation medium.

Regarding the age of the inocula used for cloning, no difference in the number of positive results was observed between the use of younger (6–12 days) or older (13–19 days) inocula.

Discussion

The results of our investigations have shown that, provided certain conditions are fulfilled, the cloning technique employing a micromanipulator is suitable for the separation of leptospiral strains from mixed cultures in which two or more different types of leptospires are present, or at least presumed to be present.

It has to be pointed out that there is a certain risk of transferring more than one cell. The danger of getting mixed cultures is particularly high when serologically different cells are isolated from the culture used for cloning. In such cases, there is a possibility that after some transfers into media without immune sera, the original type of cells becomes prevalent even though, according to the results of the agglutination test, its proportion in the cloned culture should be minimal. This agrees with the observation of Magliocchetti-Lombi and Babudieri (1968) and is most probably due to the better adaptation of the original strain to the culture medium. To avoid this danger, cultures in every well-grown tube are serologically checked. The cultures proving to be mixed are discarded and the serologically homogeneous cultures are recloned.

The last grown tube was regularly used for recloning. In all cases the recloning was performed twice, even if the clones were obtained from an apparently homogeneous culture. In this way leptospiral cultures were obtained whose serological properties did not change even after several years.

Because a part of the clones with new antigenic properties grew very poorly and the cells died within a short time, regular microscopic control and, if necessary, early transfer are considered to be important.

Cloning with a micromanipulator appears to be a suitable procedure for the separation of cultures with different properties (serological, biological, etc.), as well as for the purification of leptospiral strains isolated from water. However, it must be pointed out that successful results may be expected only if the different cells in the culture used for cloning are present in relatively equal proportions. In cultures where the proportion of one type of cell is many times greater than that of another, the probability of isolating both types is very low. In this case, in so far as the two types of cell differ in their antigenic structure, a pretreatment of the culture with hyperimmune sera, as recently proposed by Babudieri (1968) may be useful. But the use of hyperimmune sera cannot replace cloning because his suggested 6 successive transfers cannot be considered as definitely leading, in all cases, to a complete elimination of one or more of the differing strains present in the cultures. Gorshanova (1969) has reported the properties of some mixed leptospiral strains (isolated from swine) containing leptospires belonging to 2 or 3 serological groups: Grippotyphosa, Pomona, Tarassovi. She established the possibility of the prolonged co-existence of these strains *in vitro* for up to 511–524 days.

A further advantage of cloning is that it also allows the separation of serologically related strains (which is hardly possible by the technique with

immune sera) as well as strains differing in other biological properties.

A relative disadvantage of the micromanipulator technique is that it is laborious. Better types of micromanipulator are believed to reduce this disadvantage and, moreover, to diminish the risk of transferring more than one cell.

In comparison with the dilution technique of cloning suggested by Babudieri (1966), the advantage of the micromanipulator method is that the selection of cells is under direct visual control, and so viable cells can be chosen for transfer.

The plate method of cloning (Larson et al., 1959; Cox, 1966) needs further investigation. The practicability of this technique is dependent on excellent solid media that are suitable for the growth of all leptospiral cultures. Moreover, the plate technique does not exclude the possibility that some colonies may arise from clumps of cells.

In conclusion, attention is drawn to the fact that the choice of whether to use this micromanipulator technique depends on the aim pursued. In some cases it is obviously preferable to other techniques; in other cases, they may be preferred.

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Relative Resistance of the Eggs of Human Schistosomes to Digestion in Potassium Hydroxide

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Digestion of tissues in potassium hydroxide (KOH) solution is a convenient and safe means of preparing egg suspensions for the counting of schistosome eggs. *Schistosoma mansoni* eggs are somewhat more resistant to digestion than are the tissues, and an accurate assessment of the number of eggs in the tissues is possible by digestion of unfixed tissues under controlled conditions of temperature, KOH concentration and time (Cheever, 1968). As similar information was not available for *S. haematobium* and *S. japonicum* eggs, the resistance to KOH digestion of these eggs was compared with that of *S. mansoni* eggs.

Materials and methods

Eight Swiss albino mice infected with an average of 5 worm pairs of *S. mansoni* (NIH-Puerto Rican strain) for 11 weeks were sacrificed and the livers divided into approximately equal portions weighing 300 mg–400 mg. Portions of each liver were fixed in 10% neutral acetate-buffered formalin for several weeks or months. Unfixed samples from the same livers were stored at −20°C. The livers of 8 golden hamsters (*Mesocricetus auratus*) infected 6 months previously with *S. haematobium* (Egyptian strain) and harbouring an average of 7 worm pairs each were similarly treated. The liver of a chimpanzee (*Pan satyrus*) infected for 7 months with 4 pairs of *S. japonicum* (Japanese strain) was divided into 16 portions of 8 g each, which were fixed or frozen as described above.

All specimens were digested in 100 ml 4% KOH solution. Unfixed tissues were incubated at 37°C. Fixed tissues could not be digested in a reasonable time at this temperature and so were digested at 56°C. In all instances, 8 tissue samples were simultaneously digested at each temperature studied for each schistosome species. The number of eggs per

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