Methods for the study of pathogenic organisms

1881 • Robert Koch

Koch, Robert. 1881. Zur Untersuchung von pathogenen Organismen. 1881. *Mittheilungen aus dem Kaiserlichen Gesundheitsamte*, Vol. 1, pages 1–48.

. . . AFTER IT HAS BEEN DETERMINED that the pathogenic microorganism is present in the animal body, and after it has been shown that the organism can reproduce in the body and be transmitted from one individual to another, the most important experiment remains to be done. This, which is the most interesting part of hygienic studies, is to determine the conditions necessary for the growth and reproduction of the microorganism. As I have mentioned earlier, this problem can only be solved with the help of pure cultures, and I do not believe it is too much to say that the most important point in all studies on infectious diseases is the use of pure cultures.

Since the importance of pure cultures has been known for a long time, it has thus been true that all who have worked in this field of infectious disease have worked the hardest to perfect methods of pure culture. The most recent results have shown that we are not even past the first groping stages in this research. At the most, people have learned to avoid the most obvious errors, and not all have even learned this.

The most important procedures that have been developed for the manipulation of pure cultures can be summarized as follows.

A sterilized container is used which has been closed with mold-proof sterilized cotton, and this is filled with a sterilized nutrient liquid of the proper sort. Then this is inoculated with material containing the microorganism which is wanted in pure culture. After suitable reproduction has taken place in this container, a sterile instrument is used to transfer a little of this to a second container. This process may be repeated a number of times. In short, this procedure is analogous to

the inoculation of an experimental animal from a diseased animal to transfer an infectious disease.

Naturally in this procedure one has to make several assumptions, of which the first is that the culture vessel is really sterile. How lightly this sterilization has occasionally been treated can be seen from the controversy between Pasteur and Bastian on spontaneous generation, and the well-known question of the former to the latter: "Flambez-vous vos vases avant de vous en servir?" * which Bastian had to answer in the negative.

Second, one must assume that the sterile cotton is really mold-proof. As Nägeli has shown, this is not always the case.

Third, it must be assumed that the nutrient liquid is both sterile and suitable for the growth of the organism in question. . . .

Fourth, it must be assumed that the substance used as inoculum contains no other microorganisms than the one desired. Even a slight contamination of the inoculum with another species which is faster growing than the organism desired will prevent anyone from ever obtaining a pure culture. Buchner has therefore developed his own method for the preparation of an initial material for his studies on the anthrax bacillus. He inoculates the nutrient medium with such a high dilution of anthrax infected material that, through calculations, it can be assumed that only one bacillus is placed in each culture vessel. Then from the characteristic macroscopic appearance of the developing culture, he concludes that he has obtained a pure culture.† [This method also has difficulties which I shall go into later.] . . .

Fifth, it has to be assumed that during the initial inoculation and also in the subsequent inoculations, that no foreign organism gets into the culture liquid from the air. This is a danger which the experimenter will find difficult to prevent with certainty, even when the protecting cotton plug is exposed to the air for only a very short time. Even if the first, second, and third transfers have been successful, the probability that the culture will get contaminated will increase with the number of transfers. In order to circumvent this eventuality as much as possible, it is customary to prepare a number of replicates, and only use those for further inoculations which appear by macroscopic or microscopic observation to be pure. Unfortunately one cannot even rely on this procedure, because the macroscopic differentiation of several cultures is very uncertain, and even the microscopic examination is fraught with difficulties, since one only knows that the very small drop of culture fluid under the microscope is free of contaminating organisms, and, as well, if the amount of contamination is small, there may be only occasional contaminants amongst the large number of organisms, and this makes them quite easy to miss. Therefore the first initiation of contamination cannot be distinguished either macroscopically or microscopically, and if one by chance uses for further inoculations a culture which is presumed to be pure but which has already become contaminated, and the contaminating organisms are able to overtake the experimental organism, then the pure culture is completely lost. The microscope will reveal in the next generation, without a doubt, that the culture is contaminated, but now it is too late, because it is impossible at this time to rid oneself of the uninvited guest.

* ["Do you flame your glassware before using it?"]

† [This is Lister's method; see page 58.]

All in all the situation with regard to pure culture techniques is quite disappointing. No one who has cultured microorganisms in the ways currently in vogue and has not avoided completely all of the sources of error that I have indicated, can complain if his results are not accepted as fact by his fellow workers. What has been said above should be heeded by the Pasteur school in its noteworthy but blindly zealous researches, since this renders it doubtful that they have obtained in pure culture the organisms of rabies, sheep pox, tuberculosis, and so forth.

As I have emphasized many times before, pure cultures are indispensable for the further development of knowledge in the field of pathogenic organisms and all that is connected with this, and a practical and exact method must, in some way, be developed. The present methods seem to me to offer no hope for a significant improvement. . . .

Therefore I have rejected completely all of the current principles of pure culture technique and have adopted an entirely new way. A simple observation which anyone can repeat has led me to this approach.

If a boiled potato is cut in half and the cut surface is exposed to the air for several hours and then placed in a moist chamber such as a moistened bell jar in order to prevent it from drying, then, depending upon the temperature of the chamber, one will find in the following day or two, on the surface of the potato, a large number of very small droplets, all of which seem to be different from each other. Several of these droplets may be white, others may be yellow, brown, light gray, or reddish, while others appear to be spread out water droplets, or half spheres, or warty. But all of these become larger in time, then appear mycelia of molds, and finally all of the droplets coalesce and the potato soon becomes obviously spoiled. If one examines these droplets under the microscope while they are still isolated, preferably after they have been streaked on a cover glass, heated, and stained, it can be seen that each droplet consists of microorganisms of one particular species. Some reveal large micrococci, others have small micrococci, in a third the cocci will be in chains. Those which have spread considerably usually consist of bacilli of various sizes and arrangements. Many consist of yeast cells, and here and there is a mold mycelium which has come from a germinated spore. There is no doubt where these different organisms have come from. Another potato is peeled with a flamed knife to remove the peeling which contains soil with bacillus spores which have not been killed by the short heating time. This piece of potato is protected from the air by placing it in a glass beaker with a cotton stopper and then incubated and observed. In this potato no droplets develop, no organisms appear, and the potato remains unchanged until it eventually dries up after several weeks. Therefore, the germs from which the droplike colonies on the first potato developed could only come out of the air. Indeed, often one can see in the center of the droplet a dust particle or piece of thread which was the carrier of the germ. These germs may be dried but still living bacteria, yeast cells, or spores. . . .

What can we conclude from these observations on colonies developing on potatoes? It is possible that two different germs may come to lie close together and develop colonies which quickly coalesce, and it is possible that one dust particle may contain more than one germ and these may

develop simultaneously. But these are probably exceptions, and most often each droplet or colony is a pure culture and remains a pure culture until it enlarges to the point that it touches its neighbors. If instead of the potato, a liquid medium of the same surface area were exposed to the air, then undoubtedly the same number and the same kinds of germs would fall as had fallen on the potato, but the development of these germs in the liquid would be different and would follow the manner which has been previously described. The motile bacteria upon dividing would separate from each other. The nonmotile bacteria would probably begin to form tiny colonies, but these would soon be separated by the movements of the motile bacteria. Some of the organisms would sink to the bottom of the liquid, while others would rise to the top. Some of the organisms which would have found places on the potato to grow undisturbed would be choked by the development of other more luxuriantly growing organisms and would never grow. In short, the whole liquid would reveal under the microscope from the beginning a tangled mixture of different shapes and sizes, which no one would mistake for a pure culture. What is the fundamental difference between the nutrient substratum which the potato and the nutrient liquid offer to the microorganisms? It is only that the potato is solid and prevents the various species, even if they are motile, from becoming mixed, while in the liquid medium there is no chance for the different species to remain apart.

How then can we make use of the advantages which a solid nutrient medium offers for the pure culture practice? A number of the colonies which had developed spontaneously on a boiled potato were spread out on other similar potato slices and incubated in the moist chamber. Within one or two days a heavy growth of the seeded microorganism had developed, and these had exactly the same characteristics as those from the original droplet. . . . All of them grew quite quickly from very small colonies of the original potato when transferred to other potatoes and appeared to be perfectly pure cultures. Extra-special precautions to prevent air contamination were not necessary here, since if a germ of another organism fell here and there on the potato, it could only develop where it fell and would slowly spread out but would never endanger the whole culture. As well, any contaminating colonies could be easily distinguished by their appearance, so that a contamination of the culture during the next transfer could be easily avoided. . . . Here therefore was a very simple method for the production of perfect pure cultures, at least for those organisms which could grow on boiled potato, and this number is not small. . . . However, bacteria which had been shown by animal experiments to be pathogenic could not be cultured on potato.

But the principle had been found, and it was only necessary to devise conditions which could be used in all cases. There would be no purpose in outlining all of the experiments which were performed, in order to find a nutrient medium like boiled potato which would suit the pathogenic organisms. I will indicate only the end result of these experiments. In its present form the technique can be used perfectly in the majority of cases where pure cultures are desired, and in time it will undoubtedly be perfected so that all cases will be included.

After I had considered that it would be hardly possible to construct a universal medium which would be equally

suitable for all microorganisms, I limited myself to attempting to use the known media and such new ones as I might develop and converting them to a form which would be firm and rigid. The most useful way to obtain this end is to add gelatin to the nutrient liquid. . . . The mixture of nutrient liquid and gelatin, which I will call nutrient gelatin for short, is prepared in the following way: The gelatin is allowed to soak in distilled water and is then dissolved by heating. Both the gelatin and the nutrient liquid are prepared at such concentrations so that when they are mixed in predetermined amounts they will give the desired concentration of gelatin and nutrient in the final medium. I have determined that the best concentration of gelatin for these purposes is 2.5 to 3 percent. . . . One can also dissolve the gelatin directly in the nutrient liquid. Gelatin generally gives a slightly acid reaction, and for this reason it is necessary to neutralize the nutrient gelatin with potassium or sodium carbonate or basic sodium phosphate, if the medium is to be used for the culture of bacteria. The neutralized gelatin is again heated, and since there is usually a precipitate formed either during this heating or the preceding neutralization, the mixture is then filtered. This filtration also removes any impurities that were present in the gelatin. In the meantime a container closed with cotton has been sterilized by heating for a long time at 150°C., and this is then filled with the medium and boiled again. The boiling requires only a short time, since it is only necessary to kill the microorganisms which were already present in the nutrient gelatin, and these are easy to kill. The spores which are present can only be killed by prolonged heating, and this cannot be done, since the gelatin then loses its ability to solidify. For the same reason, it is not possible to sterilize with steam under pressure.* During these manipulations the nutrient gelatin is therefore not sterilized with certainty, but this makes no difference. If the medium were liquid, the spore-forming bacteria would quickly grow and spread throughout the whole liquid, and only reveal themselves through a turbidity on the second or third day. At this time the liquid could no longer be saved, since it would be changed from its original composition, and probably would be full of newly-formed spores. But in the nutrient gelatin the situation is quite different, and here can be seen already the tremendous advantage offered by the solid characteristics of the medium for revealing its content of bacteria. In the next day or two one may see dispersed throughout the transparent, solidified gelatin, a number of very small, translucent little dots, which appear white by reflected light. If one allows the nutrient gelatin to incubate further, then these little dots will soon enlarge into small spheres, and these will continue to increase in circumference, and eventually liquefy the gelatin and convert it into a turbid liquid. These small, white colonies consist of bacilli, which fact can easily be ascertained by a microscopic examination. But if one is aware of this and wishes to sterilize this gelatin, one should not wait until they have achieved such a considerable size, but kill them through boiling of the gelatin when they are just big enough to be seen by the naked eye. Here is a great advantage of the nutrient gelatin, since one cannot overlook the very first beginnings of bacterial development. . . . One discovers quickly if this or that particular nutrient fluid

* [Nutrient gelatin can be steam-sterilized if higher concentrations of gelatin are used.]

when converted into nutrient gelatin is easy or hard to sterilize. Many, as for example alkaline urine or Pasteur's fluid, are easy to sterilize in the form of nutrient gelatin. Others like meat extract or hay infusion are much more difficult; one has to boil them daily for several days. This is because not all of the spores germinate at the same time. Occasional single colonies will develop in the center of the gelatin even days after the last boiling, and their position shows that they were in there from the beginning and did not arrive later. However, if this should be the case, frequent examination of the nutrient gelatin in the first week will allow one to notice these early enough and they can then be killed by another boiling. This frequent examination in the first week should never be omitted.

Because it is so simple and certain to prepare pure cultures using potato slices, I have preferred to prepare the nutrient gelatin in a similar form as a potato slice. It can be poured into flat watch glasses, small glass plates or the like. However, the most useful for the preparation of cultures, and especially for the microscopic examination of these, is to spread the nutrient gelatin as a long, wide drop on a microscope slide, in which form it can be placed under the microscope when so desired. This is done with a previously sterilized pipette, and of course the microscope slide is previously cleaned and sterilized by prolonged heating at 150°C. The drops are about two millimeters thick. The gelatin hardens in a few minutes and the slides are placed on a small glass shelf which will hold two or three slides next to each other. Finally a number of these shelves are placed in layers over each other and placed in a moist chamber. . . . Under such conditions the gelatin drops can be kept two or three weeks before they dry out. The organism to be cultured is seeded by taking a flamed needle or platinum wire, picking up a very small quantity of the liquid or substance containing the organisms, and streaking this in three to six cross lines on the gelatin surface. . . . The expression "inoculation" for this operation seems appropriate. . . .

The bell jar which serves as the moist chamber is sufficient protection from contamination, even though it does not fit tightly. It sometimes happens that foreign organisms may fall on the gelatin during inoculation or manipulation of the slides. But these can only develop at the place on the gelatin where they have fallen and this is usually not on the inoculation streak. It is hardly possible that all of the cultures of an organism will become contaminated so that they cannot be transferred further, and this possibility is even more reduced if the bell jar is not opened often. Within a few days the pure cultures have developed to their maximum extent and can be inoculated further. There is no purpose in allowing the cultures to stand a long time, and this is especially true when the bacteria being cultured are able to liquefy the gelatin, or when sporulation has set in. In these cases a quick transfer is necessary. If it is necessary to keep single cultures for a long time without transfer, then it is necessary to keep them in a container enclosed with cotton. . . .

At low temperatures the development of the cultures proceeds quite slowly, and many organisms require a certain warmth in order to proliferate well. The most luxuriant growth in gelatin cultures has been at 20–25°C., and I have not found any organisms yet which are at all culturable, which could not grow at this temperature. However, if it is necessary to use tem-

peratures over 30°C., where the gelatin is fluid, then one cannot use gelatin or must modify the procedure. . . .*

A very important operation in the pure culture procedure is the procuring of a completely pure material for the first inoculations. This can be easily performed with the help of nutrient gelatin. With the previous methods this problem was almost impossible to solve. If, for example, blood from a septicemic animal was to be used as culture material to obtain a completely pure culture of the septicemia bacteria, previously many precautions of sterile procedure would have to be taken to remove the blood from the animal, and still the desired result would not be obtained. Now it is only necessary to take a flamed needle and remove some blood from the opened heart or a convenient blood vessel and streak it a few times on the nutrient gelatin. There will occur growth in colonies of several types of microorganisms, among which will be a greater or lesser number of pure, characteristically matlike and granular colonies which can be characterized under the microscope as those of the septicemia bacteria. It will be quite easy to culture these further in pure culture. In this case the number of foreign organisms is at a minimum, so that it is quite easy to isolate the pure colonies of the appropriate organism. However, even if this situation were reversed and the sought-for organisms were in the minority, it would still be possible to have success. Although here it would not be as easy, it would be just as certain. It is only necessary to dilute the bacterial mixture considerably and then make a large number of streaks. In such circumstances it is advantageous to inoculate into the still liquid gelatin, in order to spread the various germs over a wide area, and then pour it on the slides and locate the colonies which develop under the microscope. . . .†

I have carried pathogenic and nonpathogenic organisms over a long series of transfers on boiled potato or nutrient gelatin, without ever once observing any noticeable changes in their characteristics. They maintain their morphological as well as their physiological characteristics, so far as one can determine these, without change through months of growth as pure cultures. . . .

In botany and zoology it is a basic rule that all living organisms which have been previously unknown, should be exactly described, named, and tentatively recorded as new species. . . . This tried and approved rule, that all new forms which deviate from each other in significant ways, should be considered as separate from each other, has remarkably been often ignored in studies on bacteria. From the very beginning of bacteriological research, from Hallier to Naegel to Buchner, right up to the present time, there has been a tendency to take all of the different kinds of bacteria and throw them into one pile, and make one or at most several species from them. If it is ever possible to show that one type of bacteria can be converted into another well-known form by merely continued culture, then is the time to consider these demonstrably related forms to be one species. Up until now this proof has not been accomplished, and there is not the slightest basis in bacteriology to deviate from this general maxim of natural science. If at the beginning too many species are assumed, this can be of no dis-

* [In the next paper, page 109, an important modification is presented which allows higher temperatures to be used during incubation.]

† [The first example of a technique known today as "pour plate."]

advantage to the science. But if a priori the utility and necessity of doing research on the different forms of bacteria are denied, making it impossible to acquire knowledge, then a door will be closed on all further research and progress in this field, and this would certainly be a tremendous barrier to the progress in this young and promising subject. . . .

It seems to me indispensable in our studies on bacteria . . . to adhere to the following concept: *All bacteria which maintain the characteristics which differentiate one from another, when they are cultured on the same medium and under the same conditions, through many transfers or many generations, and which seem to be different from each other, should be designated as species, varieties, forms, or other suitable designation.*

Comment

If I had to choose one paper as most significant for the rise of microbiology, this would be it. Koch presented a method for isolating pure cultures that is so simple, reproducible and understandable that it could be performed by anyone. The development of this method led to the isolation and characterization, during the 20 years after 1881, of the causal organisms of all of the major bacterial diseases which affected mankind.

So far as I know, it is not recorded how Koch happened to make his original observations of colonies developing on potato slices. But it could have easily happened that he observed them accidentally while performing other experiments. He already knew the importance of pure cultures (see page 99) and knew that methods for developing them must be worked out. Watching the colonies develop on potatoes, suddenly everything become clear. He had his method, so, as he says ". . . the principle had been found, and it was only necessary to devise conditions which could be used in all cases." He had only to take his known liquid media and devise ways of making them firm and rigid.

Koch saw the advantages that the use of solid media would have for research on infectious disease. These advantages are well outlined in the present paper. But in addition, he saw the implications of his technique for basic bacteriology, for the concept of speciation. It was obvious that different colonial forms developed on the solid media. These colonial forms bred true and could be distinguished from one another by their colony characteristics. They also differed microscopically and in temperature and nutrient requirements. Although Koch was trained as a physician, he realized that these forms met all the requirements that botanists and zoologists set up for the delineation of species. It seemed quite reasonable to him that each form was a separate species, or variety, or other suitable designation. This idea had met with resistance in the past, before it had been possible to culture bacteria on solid media and observe their colonial forms. After the present paper was published, such resistance disappeared quickly, because it was possible for all workers to observe the distinctiveness of various bacterial forms on solid media and convince themselves that they were really separate species. When only observations in liquid media were possible, it was not possible to shed light on the controversy. So Koch's method for solid media cultivation had a tremendous impact on the young science of bacteriology, as well as on the whole field of medicine.