THE TECHNIQUE OF VIRUS CULTURE ON CHORIO-ALLANTOIC MEMBRANE IN MILITARY LABORATORIES.

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Nearly thirty years ago Rous and Murphy (1911) employed the chorio-allantoic of the developing chick in their study of fowl tumours, and subsequent workers adopted the method to the investigation of other types of neoplasms. Woodruff and Goodpasture (1931) were able to demonstrate that viruses could be cultured satisfactorily on egg membrane. Their early work was concerned with the virus of fowl-pox, but a later paper by Goodpasture, Woodruff and Buddingh (1932) reported the successful culture of the virus of vaccinia and directed attention to the potentialities of the method in producing anti-smallpox vaccine. These findings were confirmed by other workers and, inter alia, Sanjiva Rao, Pandit and Shortt (1936) were able to culture the vaccinia virus at the King Institute, Guindy. Of particular interest to the military medical authorities was the extension of the method to the viruses of dengue and sandfly fever by Shortt, Sanjiva Rao and Swaminath (1936).

For the last two years the method has been practised successfully at the District Laboratory, Peshawar, by the Peshawar Section of the Sandfly Fever Inquiry, during which period sera from a very large number of cases of suspected sandfly fever in the wards of the British Military Hospital have been investigated for the presence of virus. Its simplicity, together with the fact that the special apparatus need not be expensive, makes it capable of adoption in military laboratories at home and abroad, either as a means
of diagnosis of conditions which are due to a specific virus, or in the investigation of cases where routine laboratory examinations are inconclusive. The indeterminate fevers so commonly met with in the tropics, offer wide scope for work on these lines, and there is every reason to suppose that co-operation between clinician and pathologist will assist in solving problems in diagnosis and may lead to the isolation of some hitherto undescribed viruses. In Europe there are numerous factors, immunological and otherwise, connected with diseases such as influenza, in which there is adequate room for further work. Compared with methods of virus propagation by animal inoculation it is certainly more economical and probably more accurate, as potential fallacies from accidental cross infection, or unexpected natural immunity, are reduced to a minimum.

The method, as used at the District Laboratory, Peshawar, was practically identical with that in use at the King Institute, Guindy, a brief account of which has already been published by Sanjiva Rao et alia (1936). Various modifications are practised by other workers, notably Burnet (1936), who claims that his method is suitable as a means of virus titration.

**Summary of the Method.**

Briefly, the technique employed consisted of the inoculation of a small quantity of suspected material (serum, etc.) on to the chorio-allantoic membrane of a hen’s fertile egg at approximately the fourteenth day of incubation; three to four days later the membrane is extracted and examined against a dark background for the presence of small whitish yellow opacities known as “lesions.” These lesions, if not required for histological examination, are stored in glycerine water and subsequently ground and used for further inoculation. This process of inoculation in series is referred to as “passaging.”

It must be noted that, although the lesions can be demonstrated to contain virus particles, it is not correct to refer to them as consisting of virus. They represent the reaction of the membrane to the presence of virus.

**Details of the Technique.**

The first essential is a regular supply of fresh fertile eggs, and in most cases it will be simplest to purchase locally. Should it, however, be possible to keep a small poultry farm attached to the laboratory, the optimum proportion of cocks to hens is 4 : 1, and the fowls should be of an exceptionally good laying strain, such as White Leghorns. If the eggs are bought from dealers, some special arrangement will be required to ensure that as many as possible are fertile and, in Peshawar, the practice was to pay for fertile eggs at slightly more than the current market rate, as notified by the Officer-in-Charge Supplies, and at slightly less for unfertile eggs. About 60 per cent of eggs thus obtained were fertile, which meant that a large number had to be discarded, but this was offset by their very low price.
Eggs are incubated at a temperature of 102°F., or 39°C., slightly higher than that used for bacteriological work. In Peshawar the incubator used was a Hearson "Champion" pattern, which had a drawer capable of containing about ten dozen eggs without undue crowding and, provided that it was housed well away from draughts, could be depended on to maintain a satisfactory temperature. An ordinary Hearson bacteriological incubator was also adapted for egg incubation by fitting the interior with flat wooden egg boxes. This incubator was of the "B1" size (the usual type in military laboratories), which has internal measurements of 12 by 12 by 14 inches, and will take five boxes measuring internally 10 by 10 by 1.75 inches, each holding two dozen eggs. The eggs rest in oval holes, measuring 1.3 by 0.9 inches, cut in the base of the boxes. To ensure adequate ventilation two holes, each 1 inch in diameter, should be cut in the side of each box. A box of the type described, but without these side holes, is shown on the extreme left of fig. 2. These boxes are placed one above the other, on shelves in the incubator, and not more than two to a shelf. A proper egg incubator is fitted with a device for increasing humidity, usually consisting of a shallow zinc tray containing a piece of well-moistened coarse mesh canvas. In the adapted bacteriological incubator, this extra humidity was produced by placing in the lower inside corners a couple of "Felix" tubes, retained in position with plasticine and kept filled with water by means of a capillary pipette, fitted with a rubber teat.

Fig. 1.—Glass chamber for egg inoculation work.
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Whatever the type of incubator, before being used for eggs, the capsule should be checked as being marked 102° F. (or 39° C.) and as being in a serviceable condition. It should be observed for several days to ensure that an adequate temperature is being maintained, thermometer readings being taken three to four times daily, and it was found a good plan to record also the temperature of the water in the jacket. Too much attention cannot be given to the proper working of the incubator, as a comparatively small drop in temperature is sufficient to kill off developing embryos and to obliterate the work of several days. The practice in Peshawar was to have temperatures read and recorded at least twice a day, at fixed times, so that any slight fall could be noted and investigated.

With the apparatus working properly, the incubation of eggs is commenced. The number of eggs to be taken in daily depends on the space available and the proportion of “fertiles”, and in Peshawar, with accommodation for up to 230 eggs, the greatest daily intake was one and a half dozen. Before placing in the incubator, each egg was marked in pencil with the date of receipt and the initials of the supplier. The drawers, or egg boxes, were removed from the incubator daily for a short time (ten minutes in tropical heat, five minutes under winter conditions), during which time the eggs were very gently turned.

Tests for Fertility.

It is beyond the scope of this paper to give a detailed account of the development of the embryo and membranes. A good summary is given by Burnet (1936), and a more detailed description by Patten (1935). Although the eggs are not ready for inoculation until, at earliest, the twelfth
day, it is possible as early as the sixth or seventh day to determine, by "candling," whether or not an embryo is present. Candling consists of examining the eggs through a viewing tube in a dark chamber against a bright light. A dark chamber of dimensions 2 by 2 feet by 1 foot 9 inches, was improvised from an upturned tea chest from which one side had been removed and hung with a pair of curtains of black photographers' cloth. The viewing tube consisted of a tube of cardboard or metal, 8 inches long and 1 1/4 inches in diameter, one end being shaped so as to fit the side of the egg. The light may consist of any sort of lamp arranged so that the beam emerges through an oval opening, 1.6 by 1.3 inches. Satisfactory results were obtained with a strong electric torch fitted with a cardboard mask containing an aperture of the size indicated. With the left hand the egg is held in the opening and the appearance of the contents, as seen through the viewing tube, noted. During this examination the egg should be rotated gently. An unfertile egg appears perfectly clear, or may show vague irregular shadows which move freely as the egg is rotated. If the egg is fertile, a sixth- or seventh-day embryo, in its most typical form, shows as a well-defined semi-opaque disc, towards the centre of which is a small denser area (the embryonic eye), and from which well-defined blood-vessels radiate, the whole appearance being reminiscent of the optic disc as seen through an ophthalmoscope. In many eggs, however, the picture is much less definite, and it is sometimes difficult at this stage to decide whether an egg is fertile or not. It should be remembered that an egg which contains a live embryo will have discernible vessels and well-marked shadows which do not move when the egg is rotated. An egg which appears a dense black is almost certain to be addled.

The incubation of such eggs as were not obviously non-fertile was continued until ready for inoculation. For most viruses of medical importance the embryo, or rather its chorio-allantoic membrane, will have reached a suitable degree of development by the thirteenth to fourteenth day, with the twelfth and fifteenth days as outside limits.

Before inoculation eggs were once more candled. At this stage an egg containing a healthy chick shows plentiful blood-vessels, and the outline of the embryo has well-defined edges which may, on careful observation, show slight spontaneous movements, although the embryo as a whole retains its position when the egg is rotated. The following points were taken to indicate embryonic death: (1) Complete absence of visible blood-vessels, (2) undue mobility of the embryo on rotating the egg, (3) absence of spontaneous movements.

With a grease pencil a small mark was made on the shell immediately over the place where the embryonic shadow was densest, this being the most suitable place for inoculation as it is furthest away from the area where the chorio-allantoic membrane is deficient on the inner surface of the shell membrane. At the same time the outline of the edges of the air-space was also marked. This is usually, but not invariably, at the blunt end.
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INOCULATION.

Close to the spot selected for inoculation a small opening in the shape of an isosceles triangle, with \( \frac{3}{4} \)-inch sides and 1-inch base, was cut. The best means of doing this was found to be with a dental drill fitted with a carborundum disc 1 inch in diameter. As recommended by Sanjiva Rao et alia (1936), when cutting the drill was operated at its slowest speed in order to cause as little injury as possible to the underlying shell membrane; if the cutting is done with care, it is possible to feel when the shell membrane is reached and a definite change in note can be perceived at the same time. Burnet recommends cutting at fast speed with light intermittent touch, but it was found that this was more liable to damage the shell membrane.

The complete thickness of the shell was cut through along the sides of the triangle, the base was only cut half-way through. By means of a camel-hair brush the cut area was covered with a layer of melted paraffin wax and the egg returned to the incubator until ready for inoculation.

As an extra precaution against contamination, some workers have recommended that the selected spot on the shell should be cleaned before cutting by swabbing with 5 per cent carbolic, followed by absolute alcohol. At Peshawar this was discontinued after some months without any apparent ill-effects.

It is possible to remove a fragment of shell at the site of inoculation by means of an entomological needle, but this is more difficult and much less satisfactory than the use of the cutting drill.

The actual inoculation and subsequent manipulations were carried out inside a glass chamber such as that illustrated (fig. 1). The base of this chamber measured 3 feet 4 inches by 2 feet 5 inches, and its roof sloped forwards, the height of the front being 1 foot 10 inches and at the back 2 feet 2 inches. Its floor consisted of a sheet of plate glass, under which were placed sheets of black and white paper. As an extra precaution a curtain of muslin may be hung across the front but, under tropical conditions, this soon became unbearably hot. The roof contained a small square of wire gauze, let into the top right-hand corner, under which a bunsen or spirit lamp could be placed without danger of cracking the glass. A small hinged door, about 1 foot square, was let into the right side to facilitate reaching for instruments, which are boiled up in a beaker of water on the bench outside. The interior of this glass chamber was sprayed daily with a weak antiseptic (3 per cent carbolic).

The material for inoculation (serum, passage virus, etc.) was put up in test tubes plugged with cotton-wool, each containing a glass capillary pipette fitted with a rubber teat; these were placed in a rack inside the chamber. During inoculation (fig. 2) the egg rested horizontally, cut area uppermost, on an egg-holder consisting of a suitably moulded piece of plasticine in a Petri dish. With a sterile cataract knife, held obliquely, the flap of shell was gently levered up, the base of the triangle serving as a hinge. In a healthy egg the shell membrane thus brought into view should be shining...
white; if it appears of a dull and greyish colour, the embryo is probably
dead. During the raising of the window the shell membrane is usually
torn but, if still intact, it should be incised very carefully with the point
of the knife and a small fragment turned back. The chorio-allantoic
membrane, which lies immediately under the shell membrane, is glistening
and transparent and plentifully supplied with vessels. If the embryo is
dead it has a green or grey colour, and its appearance may be described as
"lifeless." 0.2 to 0.3 c.c. (3 to 5 drops) of inoculating material were then
dropped on the chorio-allantoic membrane from the capillary pipette. If it
tended to lie on the shell membrane and not run in readily, the membrane
was lifted up carefully with the point of the knife and the fluid was found
to run in by suction; occasionally it was necessary to enlarge the opening
slightly. The flap of shell was then replaced and covered with melted
paraffin and, with a grease pencil, the egg was marked with the date and
number of the inoculum and returned to the incubator. Before inoculating
the next egg the cataract knife was placed in boiling water for a few minutes.
For convenience, it was customary to inoculate a number of eggs at a time
and, while eggs may be kept at room temperature for up to thirty minutes
or more, care was taken that they should not be unduly exposed to cold
draughts during this time.

Eggs which have been discarded as unfertile, or otherwise unsuitable
for inoculation, may be utilised for practising the technique of cutting and
inoculating, using plain water. Preliminary experience of this kind is of
great assistance in reducing the amount of trauma on the embryo.

THE OPENING OF EGGS AND THE COLLECTION OF LESIONS.

In the case of certain viruses (e.g. that of vaccinia) which are toxic to the
embryo, or which tend to produce necrosis of the chorio-allantoic membrane,
it is necessary to open the eggs on the third day, but in most cases opening
is performed on the fourth day after inoculation.

This procedure was also carried out inside the glass chamber (fig. 3).
The instruments are first boiled up in a beaker and laid out on sterile dishes
within the chamber. Porcelain staining dishes were used for this purpose
and were sterilized either by boiling or flaming. If the instruments are laid
out as shown, with their points away from the operator, and each is returned
to the boiling water immediately after use, there is no necessity to re-
sterilize the dishes after each egg is opened. The egg was placed in the
holder, the marked air space being uppermost, and cleaned with 3 per cent
carbolic, followed by absolute alcohol, and flamed. With an egg breaker
(the handle of a 6-inch dissecting forceps) the shell was cracked and chipped
away. A circle of shell membrane was cut away with curved fine pointed
scissors and the embryo extracted into a Petri dish. As a rule it will fall
out freely, but a little assistance with the points of the scissors may be
required; it is necessary to cut across the attachment of the embryo to the
membrane and also any other adhesions that may be present, or the mem-
braine will follow the embryo into the dish. If the manoeuvre is carried out properly the membrane will now be seen lining the inner surface of the shell and the triangular cuts can be clearly seen. With straight fine-pointed scissors two cuts were made (fig. 4) through the membrane about \( \frac{3}{4} \) inch clear of either side of the site of inoculation and carried nearly to the pointed end. The proximal end of the portion of chorio-allantoic membrane between these cuts was grasped with a pair of fine pointed forceps and the chorio-allantois drawn out gently and deposited in a clean Petri dish. The portion between the cuts, which contained the lesions, if any, was examined in 50 per cent glycerine water, using the convex surfaces of two curved fine-
pointed forceps, with which the membrane can be examined without risk of injury. Against a dark background lesions are readily perceived. Their appearance varies considerably with different viruses, but they can be described as distinctly thickened greyish-white or yellowish-white opacities. In some cases they appear as a single large opacity, with firm edges, measuring up to 10 mm. in diameter and 1 to 2 mm. in thickness, and in others as a large number of small scattered foci, 1 to 3 mm. in diameter. The extent of lesion production is usually recorded by the symbols L+ + + +, L+ + +, L+ +, L+, L±, the last of which is used to designate a lesion which, either through its small size or atypical appearance, is "doubtful." The symbol "L" indicates an absence of lesions. If required for further inoculations the lesions were cut out, together with about 1⁄8 inch of membrane about each, placed in sterile test tubes and covered with 50 per cent glycerine water, in which they were stored, in the ice chest, until ready for grinding. At the site of inoculation may usually be seen two thin white lines, corresponding with the cuts which were made in the shell, along which there is a very slight thickening. These are referred to as "marginal lesions," and are not due to the presence of virus. They represent the membrane's response to trauma and serve to indicate the site of inoculation. If there is an absence of true lesions in their vicinity, the membrane can be reported as "L-.")

It was found that it was by no means unusual, when a batch of eggs was inoculated with virus-containing material, to find that one, or more, of the batch failed to show lesions. From this it follows that the absence of lesions from one membrane is not a proof of the absence of virus from the inoculating material. The standard adopted in Peshawar was to regard the absence of lesions from at least three membranes as presumptive of the absence of virus from the inoculum. It also happened that some eggs, although apparently healthy when inoculated, were found on opening to contain a dead embryo. In order, therefore, to ensure a conclusive result, at least five and, if possible, six eggs were inoculated in each batch.

**Grinding of Lesions.**

This was also carried out in the glass chamber with full precautions against contaminations. The usual practice was to grind lesions, either in pairs, or all the lesions in one batch together. The tube containing the lesions in glycerine water was emptied into a Petri dish and, with a sterile forceps, the lesions were picked up and transferred to a sterile pestle and mortar. A little nutrient broth, papaine broth, or Tyrode solution was added in the amount of approximately 2 c.c. to each lesion, together with a small quantity of glass sand (the equivalent of three to four "knife points") and the lesions ground thoroughly. After grinding, the liquid portion was transferred to a sterile test tube by means of a capillary pipette and stored in the ice chest. This represents "passage virus" which is used for further inoculations, either undiluted or with the addition of an equal quantity of distilled water.
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Neutralization Tests.

It has been shown that convalescent serum from many of the diseases produced by viruses possesses antibodies against the causative virus. The method used in Peshawar for demonstrating this antibody effect was that described by Shortt et alia (1938), by inoculating eggs with a mixture of convalescent serum and virus and noting whether the lesion producing power of the virus appears to be neutralized. The convalescent serum was first tested by egg inoculation to ensure that, of itself, it produced no lesions. Equal quantities of serum and virus (about 16 drops of each) were then mixed and left in the incubator at 37° C. for thirty to forty minutes, after which the mixture was used to inoculate eggs. Control inoculations were also carried out, using as an inoculum either pure virus or a mixture of normal serum and virus. The absence of lesions from the test eggs and their presence in the controls was evidence that the convalescent serum possessed a neutralizing power.

The Histology of Lesions.

If required for histological examination, the membrane is spread out in the Petri dish and examined without the addition of glycerine water. If lesions are present they should be covered at once with fixative fluid and subsequently double-embedded in celloidin and paraffin and sectioned at right angles to the surface (Shortt et alia, 1936). If it is desired to make a detailed study of the histology of the developing lesions, a large batch of eggs is inoculated, from which one or more eggs are opened at daily (or more frequent) intervals over four to five days and the lesions extracted, fixed, and sectioned.

Only a brief reference can be made to the actual histology of the lesions, but the changes seen appear to vary only very slightly with different viruses. The chorio-allantoic membrane may be taken as consisting of three layers, from without inwards, i.e. ectoderm, mesoderm, and entoderm. When a lesion is sectioned all three layers show thickening and proliferation of their cells in which inclusion material can be demonstrated by suitable staining methods. The outermost portion of the ectoderm may be necrosed off in the manner of a small ulcer. At present it appears doubtful whether any virus may be recognized, with certainty, from the microscopical appearance of its lesions.

Lesions Produced by Other Causes.

It will not be out of place to refer to the question of "pseudo-lesions," i.e. lesions produced by factors other than the presence of viruses. Pandit, Sanjiva Rao and Shortt (1938) investigated the response of the chorio-allantoic membrane to inoculation with a large number of substances of various kinds, and found that aluminium gel alone of the inert substances, and bacteria, produced lesions in eggs which were similar to those produced by known virus infections. The evidence to date suggests that the great majority of lesions produced by the inoculation of serum, and other body
fluids, are due to the presence of a virus. Those produced by other causes are mostly due to trauma or bacterial infection, of which the traumatic lesion is small in size and is not "passagable," i.e. if ground and inoculated into other eggs no lesions will be produced. The lesions mentioned above as being produced by aluminium gel were not passagable. Bacterial lesions, provided that the infection is not powerful enough to kill the embryo outright, usually appear as irregular soft-edged thickenings, varying considerably in appearance in different eggs in the same batch, and from which cultural tests will usually reveal the contaminating organism. Bacterial lesions are passagable.

It thus appears that if lesions are produced by serum from patients suffering with fever and these lesions conform to the appearance of virus lesions, are sterile on test and are passagable, there is fair evidence that the disease is due to a virus. More definite proof is obtained if, from subsequent tests, the virus is found to be neutralized by convalescent serum collected after an interval of six weeks, or more, from the date of onset of the original attack.

**Commentary.**

It has not been possible to give more than a brief introduction to the culture of viruses on egg membrane, but it will be realized that the potentialities of the technique cover a very wide field. Reference has already been made to its possibilities as a means of diagnosis and in the investigation of diseases of doubtful etiology. Even more important, if still somewhat remote, is its possible use in the production of antigen for immunization against virus diseases. Only one of the many methods of inoculation is described, and little has been said of the specific macroscopic and histological appearances produced by different viruses. For further details of these and similar matters, reports published by workers on various virus diseases should be consulted. References to some of these are included in the text, and particular attention is directed to the papers of the Guindy workers and to Bünnet's admirable monograph. For those who wish to pursue the subject of the propagation of viruses by tissue culture, a concise account of the method is given by Cameron (1936).

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REFERENCES.


APPENDIX.

Suggested List of Equipment for Egg Inoculation Work.

(N.B.—This list does not include articles normally available in a laboratory, such as ordinary glassware, bacteriological media, etc.)

Glass chamber—as described in text.
Dark chamber—as described in text.
Viewing lamp—as described in text.
Viewing tube—as described in text.
Incubator, fitted with egg boxes if necessary.
Cutting drill (Dental—A.C. and D.C. motor), with fourteen inch bracket and fitted with carborundum discs, 1 inch diameter.

Pestles and mortars, 3.

*Cataract knives, 4.
Scissors, straight, fine pointed, 4½ inch, pairs, 2.
Scissors, curved, fine pointed, 4½ inch, pairs, 1.
Forceps, straight, fine pointed, 4 inch, pairs, 1.
Forceps, curved, fine pointed, 4 inch, pairs, 2.
Forceps, straight, blunt pointed, 6 inch, pairs, 2.
Paraffin wax (60° C.).
Camel hair brush.
Carbolic acid (3 per cent.).
Absolute alcohol.
Glycerine water (50 per cent).
Glass sand.

*Cataract knives which have become unserviceable for surgical work may be used for this purpose. In India a supply of such knives can, almost always, be procured from the local Civil Hospital.