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The Laboratory Differentiation between Variola Major and Variola Minor

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Three methods have been used to differentiate between the viruses of variola major and variola minor in the laboratory. In this paper these three methods are compared directly, using the international reference strains of the two viruses. Results emphasize the great importance of temperature in determining the growth and spread of these viruses in the chick embryo. On the basis of the results described and of previous experience a simple diagnostic test, applicable to infective material obtained directly from the patient, is recommended. This test will enable a differential diagnosis to be made within two days of the receipt of suitable specimens. It is based on the fact that variola major virus will produce pocks on the chick chorio-allantois in eggs incubated at 38.25°C, whereas variola minor will fail so to do.

The chief difference between the two forms of smallpox that exist throughout the world has root in their virulence for the human host. Variola major has an over-all case mortality of approximately 20%, although a mortality rate of 40%-50% may be reached in unvaccinated patients. Variola minor, or alastrim, has a case mortality of 1% or less. In endemic areas, or when extensive outbreaks occur, this difference in mortality is sufficient to indicate which type of the disease is present. However, there may be no deaths, even in the variola major outbreaks, when there are only a few cases, especially if the patients have previously been vaccinated. The differences in the clinical picture are then insufficient to enable a diagnosis to be made between the two types of smallpox. In these circumstances it is extremely important to know which type of smallpox is active in the community. For this reason attempts have been made to find differences which may be demonstrated in the laboratory between the two kinds of variola virus. This paper is concerned with the methods found to be successful in determining the nature of a strain of variola virus. Experiments designed to correlate the results of various methods are reported.

Helbert (1957), working in this laboratory, studied five strains of virus from known outbreaks of variola major and five strains isolated from cases of alastrim. He tested them for virulence in chick embryos, mice and rabbits. In mice and rabbits the differences in virulence between the strains of the two types were so slight as to be of no practical value. Chick embryos showed a constant difference in mortality when they were inoculated on the chorio-allantois with comparable doses of either variola major or alastrim virus. It was found that variola major strains were more virulent than the alastrim strains in all experiments. Helbert also estimated the amounts of virus in the livers of the chick embryos and found that those infected with variola major strains yielded much more virus. The temperature at which the eggs were incubated was 35°-36°C. Andres et al. (1958) used Helbert's method to identify the virus from a solitary case which occurred in Germany.

Dinger (1956) studied seven strains of variola major virus and 27 strains of alastrim virus. He inoculated the chorio-allantois of 10-day-old chick embryos with virus and incubated the eggs for six days at a temperature that is not recorded in his paper. At the end of this period membrane extracts, diluted 500-fold, were inoculated on the chorio-allantois of 8-day-old chick embryos. From all the membranes inoculated with variola major, virus was readily recovered by this technique; with 15 of the 27 alastrim strains, some virus was recovered, but usually in small amounts.

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Nizamuddin & Dumbell (1961) observed that the growth of the two types of variola virus on the chick chorio-allantois was influenced by the temperature of incubation. They studied 14 strains of variola major and 11 strains of alastrim virus. All the variola major strains produced characteristic lesions when the inoculated eggs were incubated at temperatures up to 38.5°C. At this temperature the pocks were rather smaller than at temperatures of 35°C and 37°C and were also less numerous. None of the alastrim strains produced any pocks at or above 38°C; indeed, at 38.25°C no virus was recoverable from the membranes 48 hours after inoculation. These experiments indicated a simple diagnostic method which, in favourable circumstances, could be used on material obtained directly from the patient. Thus, a differential diagnosis could be made within two days.

It appeared that these three methods had a common basis in the greater power of variola major virus to infect, and multiply in, the chick embryo. In order to establish the interrelation of the three methods, temperature studies were undertaken on one strain each of variola major and alastrim viruses. The mortality of the embryo, the appearance and number of the lesions on the chorio-allantois and the quantity of virus recoverable from it were assessed at each of three temperatures for periods up to seven days. The results of these experiments are reported below.

MATERIALS AND METHODS

Virus strains

The international reference strains of variola major (Harvey) and variola minor (Butler) were chosen for this study (see Fenner & Burnet, 1957). Harvey was used in the third, and Butler in the fourth egg passage from the original isolations. The approximate titre of these suspensions was known from preliminary titrations.

Temperature of incubation

Fertile eggs were obtained from crossings between White Leghorns and Rhode Island Reds. They were incubated in a commercial hatching incubator, with automatic hourly rocking for 12 days at 38°C and were then prepared for inoculation by a modification of the usual method (McCarthy & Dumbell, 1961).

Inoculated eggs were kept in incubators provided with internal fans to reduce the variation in temperature over the shelf area required for each experiment. The thermostatic controls operated under a differential of 0.25°C. Provided that fairly rapid cycling of the heating circuit is obtained, the temperature fluctuation of the eggs is much less than that of the incubator itself. In preliminary experiments, sensitive thermometers placed inside each of a group of eggs, showed that the internal egg temperature remained constant and close to the maximum temperature of the air in the incubator. In the experiments recorded below, the temperatures given are those of the air in the incubators.

Pock counts

For pock counts fertile eggs were inoculated with that concentration of variola major or alastrim virus which would be expected to give approximately 200 pocks at 35°C. Groups of eggs were then incubated at 35°C, 37°C and 38.5°C. After 48 hours the membranes were excised, washed in 2% formalin and spread out in a black dish. Pocks were counted in a good light.

Embryo mortality

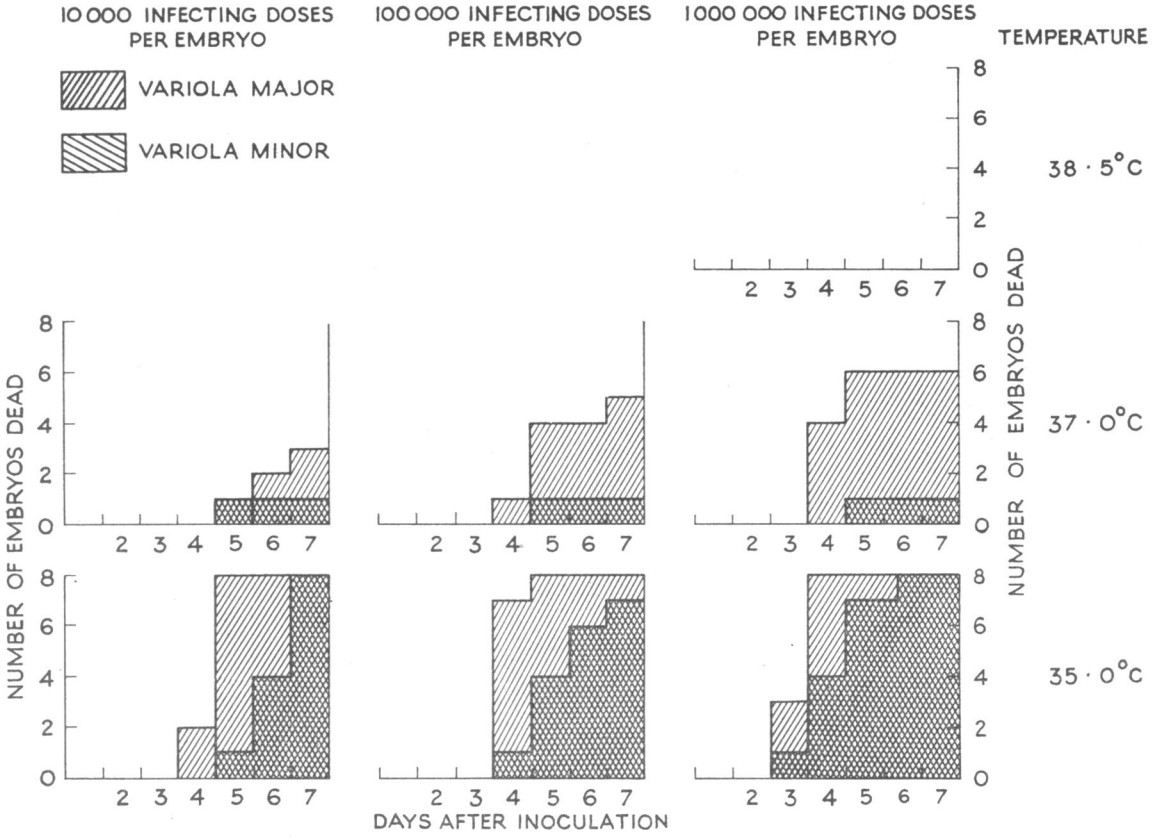
Fatalities were recorded in 12-day chick embryos inoculated on the chorio-allantois with 10 000, 100 000 and 1 000 000 pock-forming doses of variola major or alastrim virus, and incubated at 35°C, 37°C and 38.5°C. At each dose level 24 eggs were inoculated and eight were incubated at each temperature. The eggs were candled daily. Those showing no active movements of the embryo were opened by chipping away some of the shell over the artificial air space and observing the embryo directly. Rarely, the embryo was found to be alive and then the hole was repaired with adhesive tape and the egg returned to the incubator. After seven days, when the experiment was terminated, all remaining eggs were opened for a final examination.

Virus assay

Estimations were made of the amount of virus which could be recovered from the chorio-allantois of eggs inoculated with 200 pock-forming doses of virus and incubated for two, four and six days at each of the three temperatures. Two or three membranes were excised and put into a 1-ounce (28.5-ml) screw-capped bottle containing about 12 glass beads 3 mm in diameter. To this was added 1 ml of 0.004-M citrate-phosphate buffer solution, at pH 7.4, for each membrane. The

FIG. 1

CUMULATIVE DEATHS OF CHICK EMBRYOS AT DIFFERENT TEMPERATURES AFTER INOCULATION OF VARIOLA MAJOR AND VARIOLA MINOR VIRUSES ON THE CHORIO-ALLANTOIS



bottles were shaken for five minutes and the suspension removed and centrifuged at 2000 r.p.m. for 10 minutes. The supernatant fluid was then suitably diluted in buffer solution and 0.1-ml quantities were inoculated on the chorio-allantois of a group of 12-day chick embryos. Pocks were counted after 48-64 hours' incubation at 35°C.

RESULTS

The accompanying table shows the individual pock counts which were obtained in this experiment. It will be seen that the numbers of lesions are not appreciably different at 35°C and 37°C for either virus. At 38.5°C the number of variola major lesions was reduced to about 45% of the count at 35°C, but alastrim gave no lesions at all. This agrees with the finding of Nizamuddin & Dumbell

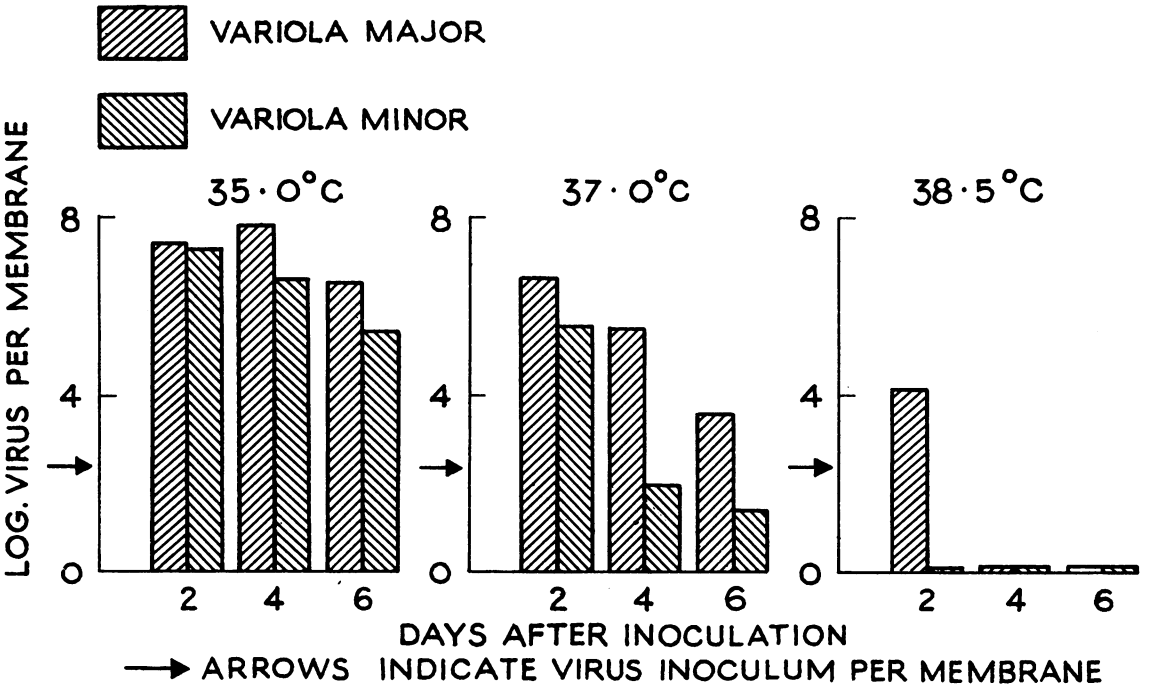
POCK COUNTS ON CHORIO-ALLANTOIS OF EGGS INCUBATED AT DIFFERENT TEMPERATURES FOR 48 HOURS AFTER INOCULATION WITH VARIOLA MAJOR AND VARIOLA MINOR VIRUSES

Temperature	Pock counts at 48 hours			
	Variola major		Variola minor	
35°C	226	241	240	310
	239	216	330	380
37°C	216	163	260	370
	146	D	340	150
38.5°C	181	36	0	0
	96	D	0	0

D = dead embryo.

FIG. 2

VIRUS RECOVERED FROM CHICK CHORIO-ALLANTOIS OF EGGS INCUBATED AT DIFFERENT TEMPERATURES AFTER INOCULATION WITH VARIOLA MAJOR AND VARIOLA MINOR VIRUSES



(1961), and, as these authors state, the variola major lesions at 38.5°C were smaller but still retained their general characters.

In Fig. 1 are shown the cumulative deaths in each group of eggs. Both variola major and alastrim were capable of killing all embryos in eggs incubated at 35°C. The deaths from variola major occurred earlier than those from alastrim but the difference was not striking, particularly at the largest dose level. At 37°C alastrim produced only an occasional death at all dose levels. These odd deaths may have been non-specific. Variola major at 37°C was demonstrably more lethal than alastrim, though 100% mortalities were not achieved. At 38.5°C, with the highest dose level, there were no deaths from either virus. It is obvious that temperature plays a dominant role in determining the mortality from these two viruses. If these results are compared with those of Helbert (1957), it would seem likely that he was using a temperature nearer to 36°C than to 35°C and that 36°C would be the optimum temperature at which to demonstrate the difference between variola major and alastrim by this technique.

Fig. 2 shows the amounts of virus recovered from membranes after different periods of incubation. At 35°C slightly less alastrim virus was recovered after four and six days' incubation, though at two days there had been no significant difference. At 37°C the distinction between variola major and alastrim is more marked. After four and six days over 100 times more variola major virus was recovered. It is obvious that a suitable dilution of the extracts made from sixth-day infections might regularly be positive for variola major and fail to contain alastrim virus. These results suggest that Dinger (1956) may have been working at a temperature close to 37°C.

At 38.5°C no alastrim virus was recovered after two, four or six days. It was noted that, though variola major had increased tenfold by the second day, no virus was recovered after four or six days. Hahon, Ratner & Kozikowski (1958) have made a similar observation for a temperature of 39°C.

DISCUSSION AND CONCLUSIONS

The experiments reported above show that variola major virus grows more vigorously in the chick embryo than does alastrim virus. They also emphasize the importance of temperature in determining the extent of virus growth. Alastrim is much more sensitive to increases in the temperature of incubation than is variola major. The upper limit of temperature at which other pox viruses will grow in the chick embryo have been determined —for ectromelia by Burnet & Lush (1936) and for vaccinia by Siim (1949). The results of a general study of this phenomenon are now in preparation (Bedson & Dumbell, 1961).

Where the temperature is so critical, accurate control is necessary. The ordinary water-jacketed incubator may not be sufficiently reliable. It is important that continuous circulation of air inside the incubator should be maintained by a fan; otherwise there may be gross local temperature variations. The best results were obtained from incubators in which a fan blew directly across heating coils and where a rapid cycling of the heating element took place. A temperature differential of 1°C might be acceptable under these conditions because an individual egg is too large to follow the rapid fluctuations in air temperature. If the shelf temperature is measured by a thermometer in a small bottle of water, a constant reading will be obtained, but the temperature so recorded will be close to the minimum air temperature. This is a convenient method but it must be remembered that if the water temperature reaches 38.5°C (Nizamuddin & Dumbell, 1961) the average air temperature will be higher than this and even variola major virus will fail to produce pocks.

For the rapid identification of a strain of virus from a suspected case of smallpox the method of Nizamuddin & Dumbell (1961) offers obvious advantages. The technique described by Dinger (1956) does not give an unequivocal answer for every single specimen and requires more than a week to complete. The estimation of chick embryo mortality (Helbert, 1957) gives a reliable answer in about 14 days but requires a large number of eggs and is very time-consuming.

In the method recommended by Nizamuddin & Dumbell (1961) it is important to run parallel tests with known strains of the two types of virus; but even when this is done, relatively small numbers of eggs are needed for the whole test. The dose of virus that it is convenient to use as inoculum is that likely to give about 200 pocks at 35°C. Two groups of three to six eggs are inoculated with each virus. One group is incubated at 35°C and the other at 38.25°C for 48 hours. If pocks appear at both temperatures the virus is variola major; if only at 35°C, the virus is variola minor.

The test can be performed with material directly from the lesions of the patient. Indeed, we have had reliable results when the inocula were extracts of crusts (3-6 crusts per ml) from cases both of alastrim and variola major. Crust extracts should be tested undiluted and at a dilution of 1/100 in order to cover the range where approximately 200 pocks may be expected at 35°C. Thus it is possible, by this method, to determine whether a case is one of variola major or variola minor within two days of the receipt of infective specimens in the laboratory.

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RÉSUMÉ

Lors d'une épidémie de variole, le taux de mortalité permet en général de dire si l'on se trouve en présence d'une forme majeure (variole asiatique) ou d'une forme mineure (alastrim).

Toutefois, il se peut qu'aucun cas mortel ne soit observé si le foyer épidémique est limité à quelques cas seulement, et cela tout particulièrement dans une population partiellement immune. Il est alors impossible d'établir un diagnostic différentiel entre les deux formes de la maladie d'après le seul tableau clinique. Dans ces circonstances, le diagnostic différentiel de laboratoire est important, ne serait-ce que pour déterminer l'ampleur des mesures préventives et de la revaccination.

Trois méthodes de laboratoire ont été proposées dans ce but: celles d'Helbert (1957), de Dinger (1956) et de Nizamuddin & Dumbell (1961). Dans ce travail, les

auteurs comparent directement ces trois méthodes, utilisant les souches internationales de référence de variole majeure (Harvey) et de variole mineure (Butler). Les résultats obtenus démontrent l'influence déterminante de la température d'incubation sur la multiplication et la propagation de ces virus chez l'embryon de poulet. S'appuyant sur ces données et leur expérience passée, les auteurs recommandent une méthode de diagnostic simple, à partir du matériel infectieux provenant directement du malade. La méthode recommandée repose sur le fait que les lésions spécifiques du virus de la variole majeure sont encore présentes chez l'embryon de poulet incubé à 38,25°C, ce qui ne se produit jamais dans le cas de la variole mineure.

La méthode permet d'établir un diagnostic différentiel entre variole majeure et variole mineure dans les deux jours suivant la réception des spécimens pathologiques.

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