

## Efficiency of the use of pock size on the chorioallantoic membrane of fertile hen's eggs as a method of typing herpes simplex viruses

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### SUMMARY

One hundred and eighteen herpes simplex virus isolates were typed in a diagnostic virology laboratory using their standard procedure by pock size on the chorioallantoic membranes (CAMs) of fertile hen's eggs. Forty-three were typed as type 1 and 75 as type 2. The isolates were then sent to a research laboratory in which they were typed blind, with or without subsequent passage in tissue culture, by neutralization with type-specific antisera. Discrepant results were found with only two isolates. The isolates were then typed by the more time-consuming but unambiguous method of restriction endonuclease analysis of their DNAs. Typing by this method confirmed the typing by neutralization and established that typing by pock size on CAMs was correct in about 98% of cases.

### INTRODUCTION

Herpes simplex virus types 1 (HSV-1) and 2 (HSV-2) can be distinguished by a number of biological, serological and biochemical methods. Amongst the biological methods are plaque morphology, thermolability and pock size on the chorioallantoic membrane (CAM) of the fertile hen's egg. Serological methods include immunofluorescence, neutralization, ELISA and radioimmune assay. Biochemical methods include studies of G+C content of DNA, restriction endonuclease analysis of DNA, comparison of polypeptides by SDS polyacrylamide gel electrophoresis (SDS-PAGE) or of enzymes such as thymidine kinase.

Some of these methods are clearly more reliable than others in giving unambiguous typing of isolates. For example, restriction endonuclease analysis of DNA or identification of polypeptides by SDS-PAGE gives unequivocal typing (Buchman *et al.* 1978; Linneman *et al.* 1978; Cassai, Sarmiento & Spear, 1975; Powell, Mirkovic & Courtney, 1977) but takes longer than one would like in a diagnostic laboratory and for speed must rely on the use of radioactive isotopes which not every diagnostic laboratory chooses to employ.

One of the methods of typing that is still commonly employed in diagnostic laboratories relies on identification of HSV-2 strains by virtue of the fact that they produce larger pocks than HSV-1 strains on the chorioallantoic membranes (CAMs)

of fertile hen's eggs. This method is fairly rapid, relatively inexpensive and employs eggs from a supply which the laboratory is probably maintaining for other purposes. The purpose of this study was to establish the effectiveness of typing HSV isolates by poek size on CAMs. Results obtained by a diagnostic laboratory which routinely uses poek size on CAMs for typing HSV isolates were compared with those obtained by a serological method (neutralization with type-specific antisera) and a biochemical method (restriction endonuclease analysis of DNA) performed by another laboratory.

#### MATERIALS AND METHODS

##### *Viruses and virus assays*

The HSV strains used in this study were 118 unselected human isolates from various sites of infection which had been sent to a diagnostic virology laboratory. HSV-1 strain HFEM (Watson *et al.* 1966) and HSV-2 strain 3345 (Sim & Watson, 1973) were used as the standard strains and for the production of antisera. All viruses were assayed in BHK cells by the suspension method of Russell (1962) using an overlay medium containing 0.8% (v/v) carboxymethyl cellulose.

##### *Cells and media*

Vero, human embryo lung and human amnion cells were used for the initial passage of viruses in the diagnostic laboratory. These cells were grown in 199 medium containing 5% (v/v) calf serum and maintained in 199+1% calf serum. BHK 21 clone 13 cells grown in Eagle's medium (Glasgow modification autoclavable powder - Autopow, Flow Laboratories) supplemented with 10% (v/v) tryptose phosphate broth and 5% (v/v) calf serum were used for the production and titration of further virus stocks, for antigen production and for virus studies involving neutralization and analysis of virus DNA.

##### *Typing of HSV strains*

Viruses were initially typed in a diagnostic laboratory using CAMs as described below; the viruses were then sent to a university laboratory where the typing was confirmed by neutralization using type-specific antisera and by analysis of their DNA. These tests were performed 'blind' in that no information regarding type of virus or site of isolation was disclosed until the serological and biochemical tests were complete.

##### *Typing by poek size on chorioallantoic membranes*

The CAMs of 10 to 12-day-old embryonated hen's eggs were inoculated with 0.2 ml of two dilutions of infected tissue culture fluid. The viruses were not titrated but a dilution giving anything between 10 and 200 poeks was found to be satisfactory. Each virus was inoculated into duplicate eggs. After incubation at 36-37 °C for between 65 and 72 h, the CAMs were harvested. The size of the poeks produced on the membrane was estimated and used to differentiate between type 1 and type 2 strains of HSV. The production of large poeks has been associated with type 2 strains of HSV and smaller poeks with type 1 strains (Nahmias *et al.* 1968). Viruses producing poeks with an average diameter of greater than 0.5 mm were typed as HSV-2, whereas viruses giving smaller poeks were classed as HSV-1.

### *Preparation of antisera*

General antisera to HSV were prepared by repeated immunization of rabbits with extracts of rabbit kidney cells infected with HSV-1 or HSV-2 as described by Watson *et al.* (1966).

*Type-specific antisera.* Antisera specific to HSV-1 or HSV-2 were prepared by serum absorption as described by Sim & Watson (1973). Briefly, to obtain type-1-specific antiserum, 1 ml of HSV type 1 general antiserum was absorbed overnight at 4 °C with sonicates of  $10^9$  HSV-2-infected cells. The mixture was then centrifuged at 105 000 *g* for 1 h at 4 °C, the supernatant frozen and thawed, heated at 56 °C for 30 min, recentrifuged at 105 000 *g* for 1 h and concentrated to 1 ml by vacuum dialysis. Type-2-specific antiserum was prepared in a similar manner by absorbing type 2 general antiserum with HSV-1-infected cells.

*Anti Band II serum.* Antiserum to the Band II antigen (gD) of HSV-1 described by Watson & Wildy (1969) was absorbed with type 2 antigen to produce a Band II type-1-specific antiserum.

*Anti VP 7/8 serum.* Antiserum to VP 7/8 region of HSV-1 was prepared as described by Powell *et al.* (1974) by footpad immunization of rabbits with appropriate polyacrylamide gel slices; the resulting antiserum was type 1 specific.

*Absorbed pre-immune serum.* Pre-immune serum from rabbits was absorbed with uninfected cell extracts as described above for the production of type-specific general antisera.

### *Typing by neutralization*

A 20  $\mu$ l sample of virus diluted to  $5 \times 10^4$  p.f.u./ml was mixed with an equal volume of each of the five antisera, and incubated at 25 °C for 3 h. Surviving virus was assayed by the suspension method of Russell (1962). Results are presented as percentage survival in the presence of antiserum, compared to that with pre-immune serum.

### *Neutralization in a 96-well plate*

One hundred  $\mu$ l of infected tissue culture fluid (from the diagnostic laboratory) were mixed with 100  $\mu$ l of a 1 in 10 dilution of either type-1-specific, type-2-specific or pre-immune antiserum in a 96-well plate for 2 h at 25 °C. One hundred  $\mu$ l from each well were then transferred to a 96-well plate (Linbro 15-FB-96) containing preformed BHK monolayers. After two days incubation at 37 °C the wells were examined for cytopathic effect.

### *Labelling of virus DNA and restriction endonuclease analysis*

BHK cells ( $4 \times 10^5$ ) were infected at a multiplicity of infection of 0.05 p.f.u./cell in the presence of  $^{32}$ P orthophosphate (50  $\mu$ Ci in 0.5 ml). Following incubation at 37 °C for 48 h, DNA was prepared and analysed with restriction endonucleases as described by Lonsdale (1979). The restriction enzymes used were Bam HI, Sal I, Hind III, Hpa I, Bgl II and Eco RI (Boehringer Mannheim or Bethesda Research Laboratories).

Table 1. *Results of typing of HSV isolates by pock size on CAMs, by neutralization with type-specific antisera and by restriction endonuclease analysis*

Site of isolation	Results of typing by					
	Pock size on CAMs		Neutralization		Restriction endonuclease analysis	
	Type 1	Type 2	Type 1	Type 2	Type 1	Type 2
Brain	6	0	6	0	6	0
Skin	7	0	7	0	7	0
Eye	1	0	1	0	1	0
Mouth	4	0	4	0	4	0
Throat	6	0	6	0	6	0
Genital (female)	3	6	2	7	2	7
Vagina	0	1	0	1	0	1
Vulva	6	13	6	13	6	13
Labia	1	1	1	1	1	1
Cervix	2	7	2	7	2	7
Urethra and urine	0	4	0	4	0	4
Genital (male)	0	13	0	13	0	13
Penis	5	26	4	27	4	27
Prepuce	2	1	2	1	2	1
Urethra	0	3	0	3	0	3
Total	43	75	41	77	41	77

## RESULTS

Table 1 shows the results of typing by pock size on CAMs in the diagnostic laboratory. Forty-three isolates were typed as type 1 and 75 as type 2.

Table 2 shows the results obtained by neutralization of the reference strains HFEM (type 1) and 3345 (type 2) with type-specific antisera as described in Materials and Methods. Type-1-specific antiserum, Band II type-1-specific antiserum and type 1 VP 7/8 antiserum all neutralized HFEM in that after 3 h, 1% or less of the virus survived; 3345, however, was not neutralized by these three antisera. Similarly type-2-specific antiserum does not neutralize strain HFEM, since 99% of the virus survived after 3 h, but it did neutralize strain 3345.

Table 2 also illustrates the corresponding results for some of the recent isolates, indicating the ease and clarity with which they can be typed as type 1 or type 2 by neutralization with these antisera. Table 1 summarizes the overall results obtained by typing the 118 isolates by this method. As can be seen, except for two of the viruses, the typing in the diagnostic laboratory was in agreement with the typing by neutralization.

Isolate numbers 2372 and 7432 were both typed as type 1 by pock size on CAMs. The detailed results of neutralization tests with these viruses are shown in Table 2, from which it is clear that they behave as type 2 viruses.

In order to establish unambiguously the type of isolates 2372 and 7432, the DNA of these viruses was digested with restriction endonucleases and the resulting fragments compared by agarose gel electrophoresis with those of laboratory-established strains HFEM (type 1) and 3345 (type 2) and a number of other recent

Table 2. *Typing of HSV isolates by neutralization with various type-specific antisera*

Percentage survival after 3 h neutralization with

Virus isolate number	Site of isolation	Percentage survival after 3 h neutralization with				HSV type by neutralization
		Type-1-specific antiserum	Type-2-specific antiserum	Band II type-1-specific antiserum	Type 1 VP7/8 antiserum	
HFEM	Face	0	99	1	1	1
3345	Genitals	81	2	78	96	2
0539	Brain	1	104	8	4	1
6225	Prepuce	5	72	7	6	1
6400	Cervix	2	97	4	2	1
6509	Throat	3	88	6	6	1
7141	Throat	1	72	9	2	1
7245	Cervix	1	90	6	9	1
2372	Penis	104	4	98	100	2
6255	Penis	95	7	102	95	2
6256	Prepuce	98	2	87	86	2
6401	Vulva	80	2	91	88	2
7432	Genitals	108	6	100	92	2
9937	Penis	78	16	98	88	2

isolates for which the typing by pock size and by neutralization was in agreement. Cells were therefore infected with strain HFEM, strain 3345 and with recent isolates, and the DNA was extracted and digested with restriction endonucleases as described in Materials and Methods. The resulting fragments were separated by agarose gel electrophoresis, and Fig. 1 shows an autoradiogram of the fragments obtained following digestion with Bam HI. Clearly the pattern of fragments obtained with type 1 strains (lanes 1 and 7–10) is markedly different from that obtained with type 2 strains. The latter lanes include isolates 2372 (lane 3) and 7432 (lane 13). In addition, however, it is clear that differences can also readily be discerned in the mobility and/or number of fragments within type 1 or within type 2 strains. These findings were confirmed by digestion with Sal I, Hind III, Hpa I, Bgl II and Eco RI (data not shown). The results of typing the isolates by restriction endonuclease analysis of their DNA are summarized in Table 1 and show complete agreement with typing by neutralization. Isolates 2372 and 7342 were subsequently retyped by pock size on CAMs and in both cases the viruses were now typed as type 2, in agreement with the results from the neutralization tests and restriction endonuclease analyses.

Since the neutralization tests give such reliable results, attempts were made to make the test more rapid. In the test as described above,  $5 \times 10^4$  p.f.u. of virus were required for each neutralization. This clearly means that any virus isolate must be passaged in tissue culture to boost the amount of virus and the stock titrated before it can be used in the neutralization test. In order to obtain a result much sooner, equal volumes of tissue culture fluid from the diagnostic laboratory and type-specific antiserum (diluted 1 in 10) or pre-immune serum were pre-incubated as described in Materials and Methods and then added directly to monolayers of cells in a 96-well plate and incubated at 37 °C for two days. Fig. 2 shows the results obtained with 10 of the viruses tested. Strain HFEM (column 7)



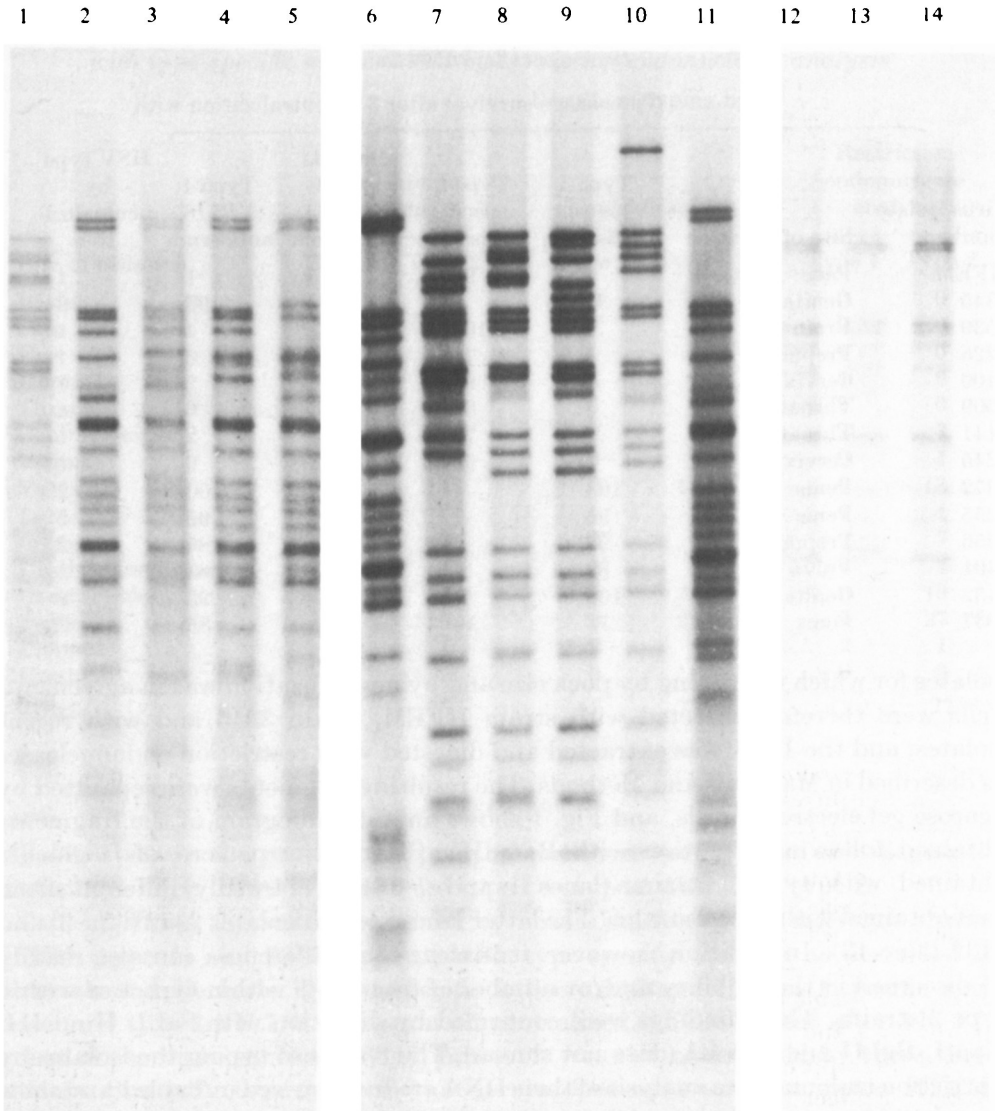


Fig. 1. Autoradiogram of  $^{32}\text{P}$  fragments separated by electrophoresis on a 0.6% agarose gel following digestion of the DNA of HSV isolates by Bam HI as described in Materials and Methods. The lanes 1-14 are from isolates 5809 (genital), 5938 (genital), 7432 (genital), 6955 (genital), 1661 (vagina), 5698 (penis), HFEM, 5809 (genital), 5839 (throat), 5941 (mouth), 3345 (penis), 2372 (penis) and 2590 (penis) respectively. The figure is a composite of parts of three autoradiograms, lanes 1-5, 6-11 and 12-14 being run together.

and the viruses in columns 1, 2 and 3 were all neutralized by type-1-specific antiserum but not by type-2-specific or pre-immune serum, and are therefore typed as type 1. The result for strain HFEM is not obvious by eye since this strain causes syncytial cytopathic effect (fusion of infected cells) and so the cell sheet, to the naked eye, is not destroyed. Use of a microscope, however, reveals the formation of syncytia in wells containing HFEM plus type-2-specific antiserum or pre-immune

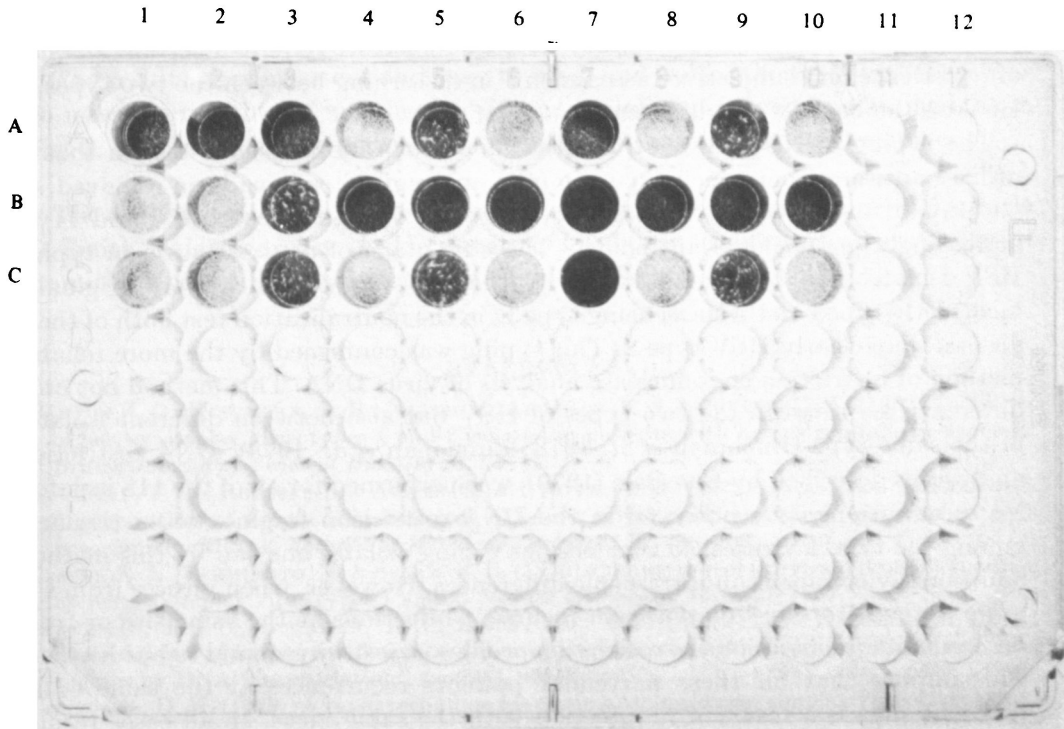


Fig. 2. Neutralization of HSV isolates with type-specific antisera. The viruses used in columns 1–10 respectively were 0539 (brain), 7141 (throat), 7245 (cervix), 9937 (penis), 6255 (penis), 6256 (prepuce), HFEM, 3345, 2372 (penis) and 7432 (genital). The top line (A) shows the results following pre-incubation at 25 °C for 2 h with type-1-specific (absorbed) antiserum; the centre line (B) shows the result following pre-incubation with type-2-specific (absorbed) antiserum and the bottom line (C) that following pre-incubation with pre-immune serum.

serum. The result for isolate 7245 (column 3) is not as clear-cut as for the isolates in columns 1 and 2 because the amount of virus present in the tissue culture fluid of 7245 was appreciably less. Incubation for a further day resulted in increased cytopathic effect in wells B3 and C3, making the typing obvious, but an earlier conclusion is possible by using microscopy. The remaining six columns show cytopathic effect with type-1-specific antiserum and pre-immune serum but not with type-2-specific antiserum. Results with columns 4, 6, 8 (strain 3345) and 10 (isolate 7432) are clear-cut, results in columns 5 and 9 (isolate 2372) slightly less so, again due to their being less virus in these two tissue culture fluids, but interpretations were unambiguous with use of a microscope, these six viruses typing as type 2, interpretation being possible by 36 h after receiving the specimens from the diagnostic laboratory.

#### DISCUSSION

As shown by the results, isolates of HSV can be satisfactorily typed by their ability to produce different-sized pocks on CAMs. Since only two of the 118 isolates were incorrectly typed, this would indicate that error had occurred in the reading

or recording of results, a mistake which inevitably could be made in any assay procedure. However, the CAM assay does by its nature require a certain amount of experience and subjective 'assessment' in discerning between the two types of HSV on the basis of the difference in size of pock.

The typing of HSV by neutralization is a much more specific test in that it utilizes antisera from which type-common antigenic sites have been removed, or antisera of more restricted specificity, e.g. VP 7/8 and absorbed Band II or increasingly, monoclonal antibodies. The advantage of neutralization in typing HSV isolates can be demonstrated by the two viruses which were originally incorrectly typed on CAMs as being type 1; in the neutralization test both of these viruses were clearly HSV type 2. This typing was confirmed by the more reliable method of restriction endonuclease analysis of virus DNA. This method not only differentiates between the two types of HSV but also between different isolates of the same type (Buchman *et al.* 1978; Linneman *et al.* 1978). DNA restriction analysis as described by Lonsdale (1979), was performed for all of the 118 isolates. Many variations were observed in the DNA restriction fragments, particularly among the type 1 viruses, so that besides typing isolates one can by this method additionally comment on intra-typic differences. However, when viruses from the same patient were re-isolated from recurrent infections at the same site or from different sites of infection the restriction profiles were always found to be identical. This implies that for these particular patients recurrences at the same or at different sites is a result of re-infection with the same virus. In all cases results of the restriction profiles confirmed the neutralization results.

Amongst the viruses in this particular study there was a higher proportion of genital than non-genital isolates; this is due to the fact that the majority of HSV isolates received in the diagnostic laboratory were from VD clinics. There is also less of a tendency for a general practitioner to take swabs from a cold sore or other non-genital lesion except in unusual circumstances, since currently, typing of isolates is of no benefit to the patient. It is evident, however, that all the non-genital isolates were HSV type 1, whereas the genital isolates consisted of both type 1 and type 2. In our present study 12% of the male genital isolates were type 1, whereas, of the female genital isolates, 25% were HSV-1, 55% of which were vulval isolates. Similar proportions of HSV-1 genital infections were reported by Smith, Peutherer & Robertson (1976), where 22% of female and 9% of male genital isolates were caused by HSV-1. However, more recent studies indicate that HSV-1 may be more involved in genital infections. Barton *et al.* (1982) have reported a type 1 incidence of 61.3% of genital infections.

The increase in genital herpes infections and the tendency of HSV-1 to be associated with genital infections demonstrates the importance of an accurate technique for identification and typing of the virus. The method of typing in CAMs, which is at present routinely used in some diagnostic laboratories, has been shown to be satisfactory for typing HSV isolates. However, is 98% accuracy good enough in the long term, when hopefully type-specific chemotherapy will be possible? In view of this the neutralization test was adapted to a small scale, suitable for use in a routine diagnostic laboratory. This method was found to be quick and easy to perform, and the results were obtained within two to three days of receiving the sample. The method does however require type-specific antiserum, the



preparation of which may be difficult for some diagnostic laboratories. In the near future, however, such antiserum is likely to be replaced by monoclonal antibodies.

The results were clear-cut and easy to read, and unlike the CAM test there was no messy harvesting of membranes. If the plaques were fixed and stained, as in Fig. 2, this would give a permanent record of the results for later reference.

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