

A simple biological marker to differentiate the types of Herpes Simplex Viruses in resource-limited settings

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Abstract

Herpes simplex viruses (HSV) multiply readily on the chorioallantoic membrane (CAM) of embryonated hen's egg and produce easily visible foci or pocks on this membrane. In the present study, pocks produced by the two antigenic types of HSV (1 & 2) were compared to evaluate the effectiveness of typing HSV isolates by pock size on CAMs. A total of 57 HSV isolates from both non-genital and genital samples were typed by the pock size produced on the CAMs of fertile hen's eggs. Twenty two HSV isolates yielded visible pocks on CAM, of which 9 (40.9%) produced small pocks, while 13 (59.1%) produced large pocks. All pocks produced on CAM were confirmed by antigenic typing by the Direct Fluorescent Antibody (DFA) method. HSV isolates which produced small pocks were in complete (100%) concordance with HSV type-1, while those producing larger pocks were in full (100%) concordance with HSV type-2. Thus, the pock size on CAM of embryonated fertile hen's egg may be used as a simple and relatively inexpensive biological marker for the differentiation of HSV types 1 & 2.

Introduction

Herpes simplex virus (HSV) can be differentiated antigenically into two groups, types 1 & 2. Type-1 is generally associated with non-genital infections while type-2 is commonly associated with genital infections, although both types may cause similar diseases at both anatomical sites¹. However, the frequency of reactivation of HSV is influenced by anatomic site and the type of virus². Genital HSV-2 infection is twice as likely to reactivate and recurs 8-10 times more frequently than genital HSV-1 infection. Conversely, oral-labial HSV-1 infection recurs more frequently than oral-labial HSV-2 infection³. It is therefore important to identify the type of HSV which cause herpetic (herpes labialis or genital herpes) infections as this influences prognosis and treatment recommendations⁴. Moreover, typing of HSV influences counseling of patients⁵.

A number of biological and serological methods are available to differentiate HSV types 1 & 2. Among these, certain biological tests are more amenable to routine diagnostic virology procedures than serological tests used to determine antigenic differences. Some techniques, e.g., buoyant density determination⁶, infectivity titers in different cell cultures⁷ and inoculation of HSV into animals such as mice⁸ are research techniques requiring special

equipments, facilities, or excessive supplies for a diagnostic laboratory. The same applies to the use of Vero cells in which type-2 HSV were markedly inhibited at temperatures greater than 39°C, whereas type-1 HSV replicated easily at 39.8 to 40.3°C⁹. Other techniques, such as determination of cytopathic effect on a variety of cell cultures may be rather subjective¹⁰.

Herpes simplex virus multiplies readily on the chorioallantoic membrane (CAM) of embryonated hen's egg and produces easily visible foci or pocks on this membrane¹¹. Many investigator were able to differentiate these two types of herpes simplex viruses by measuring the size of pocks on CAM. Larger pocks produced on CAMs has been associated with type 2 HSV and smaller pocks with type-1 HSV¹²⁻¹⁶. In the present study, clinical samples were used to determine the relationship of pock size on CAM with the two antigenic types of HSV isolates.

Materials and Methods

This randomized study was conducted at the Department of Virology, Bangabandhu Sheikh Mujib Medical University (BSMMU) from July 2008 to June 2009. Samples were collected from vesicular and non-vesicular lesions of 57 clinically

diagnosed herpetic lesions (genital and non-genital) by polyester-tipped applicators. After collection, the swabs were immediately immersed in 1 ml of Eagle's minimal essential medium containing 400 U/ml of Penicillin, 100 µg/ml of Gentamycin, 10 µg/ml of Amphoterecin-B and 2% fetal bovine serum. The specimens were frozen at -70°C temperatures within 30 minutes of collection.

For egg inoculation, 0.1 ml of specimen was inoculated on the CAM of 10-12 days old embryonated hen's egg using the false air sac technique¹⁷. Each sample was inoculated into 3 eggs. Known type-1 and type-2 strains were included as control. After incubation in the egg incubator (Mashalles, Type G-180, Spain) at 37°C for 3 and 5 days, the CAMs were harvested. The harvested membranes were placed in a Petridish containing normal saline to detect the characteristic lesions or pocks. The size of the pocks was estimated with an ocular micrometer mounted on a light microscope. Pock size was divided at 3rd day of inoculation into two groups based on the following criteria:¹²

Small pocks: Commonly produced by HSV-1, having an average diameter less than 0.5 mm.

Large pocks: Commonly produced by HSV-2, with average diameter greater than 0.5 mm.

On the 5th day, pock size which becomes larger as compared to 3rd day were designated as HSV-2, but those that remained typically small were designated as HSV-1¹⁴. Measurement of pocks were taken before antigenic typing.

For antigenic typing of HSV, membranes were ground with mortar and pestle in PBS (pH 7.3) and spotted onto a slide for identification and typing of HSV by the direct fluorescent antibody method (DFA) (Pathfinder HSV type-I & type-2, Bio-rad, India, Cat no-25215). A positive immunofluorescence result was indicated by the presence of one or more intact cells exhibiting typical appearance of apple green fluorescence. Typing was determined by the appearance of a specific fluorescent reaction with one of the monoclonal antibodies. HSV-1 infected cells showed cytoplasmic staining, while HSV-2 infected cell showed both cytoplasmic and nuclear staining.

Results

Out of the total 57 samples tested, 22 isolates produced visible pocks on the CAMs, of which 14 (63.6%) isolates were obtained from genital sites and 8(36.4%) were obtained from non-genital sites.

Of the 14 samples from genital sites, 13(92.9%) produced large pocks on the CAM at 3rd day with an average diameter of 0.5 - 0.9 mm (Fig-1) and become larger (2-3 mm) at 5th day (Fig-2). Only one (7.1%) HSV isolate produced small pocks (<0.5 mm) at 3rd day and its size remained typically small at 5th day. All 13 genital isolates which produced large pocks were confirmed as HSV-2 by DFA method while only one genital isolates which produced small pock was confirmed as HSV-1 by the DFA method (Table-I).

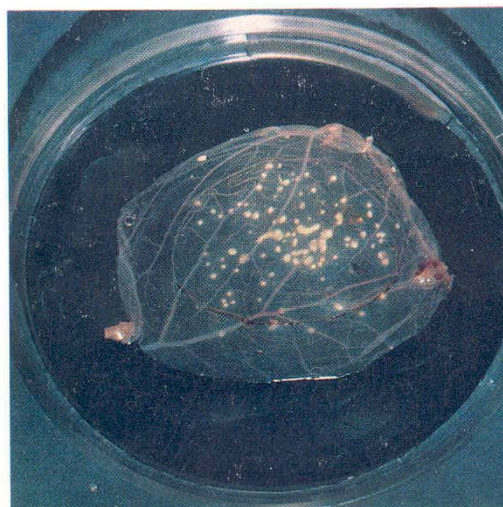


Fig-1: Pock produced by HSV-2 on 3rd day.

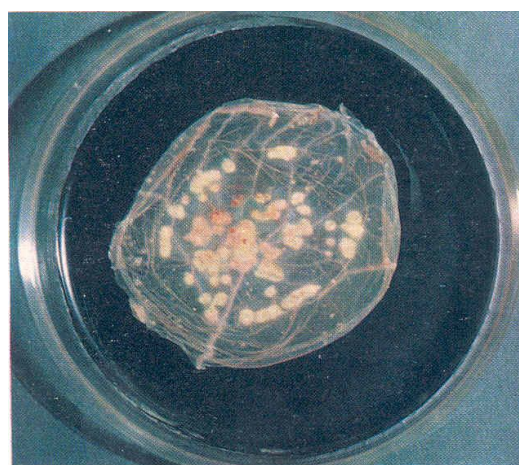


Fig-2: Pocks produced by HSV-2 at 5th day (size has increased).

Table-I: Results of typing genital isolates of HSV by size of pocks and DFA method

Sex	No. of isolates (n=14)	Site of lesion	Size of pocks on CAM	Type of HSV (by DFA test)
M	3	Inside urethra	Large	Type-2
M	4	Shaft of penis	3 Large 1 small	Type-2 Type-1
M	3	Glans penis	Large	Type-2
F	1	Vagina	Large	Type-2
F	3	Vulva	Large	Type-2

Out of the 8 isolates obtained from extra-genital sites, all produced small pocks at 3rd day with an average diameter of 0.2-0.5 mm (Fig-3) and showed no change in size of lesion at 5th day (Fig-4). On typing of non-genital isolates in relation to pock size, all were confirmed as HSV-1 by the DFA method (Table-II).

Thus, a total of 9 (40.9%) samples were identified as HSV type-1 and 13 (59.1%) as HSV type-2 according to size of pocks on the CAM of embryonated hen's egg.



Fig-3: Pock produced by HSV-1 on 3rd day.

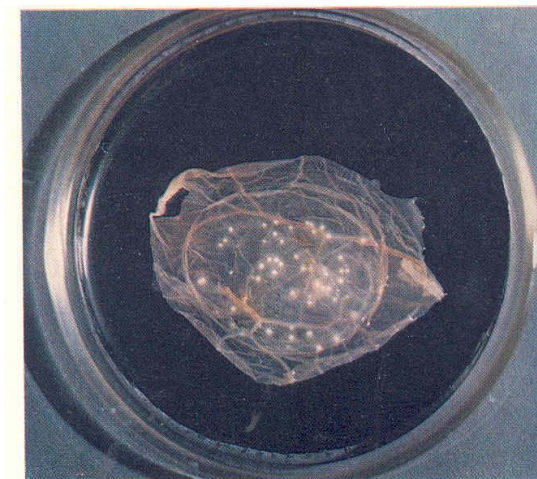


Fig-4: Pocks produced by HSV-1 at 5th day (size remained same).

Table-II: Results of typing of non-genital isolates of HSV by size of pocks and DFA method.

Sex	No of isolates (n=8)	Site of lesion	Size of pocks on CAM	Type of HSV (by DFA test)
F	4	Herpes labialis	Small	Type-1
F	2	Acute gingivostomatitis	Small	Type-1
M	1	Herpetic whitlow	Small	Type-1
M	1	Herpes labialis	Small	Type-1

Discussion

It is possible to type HSV isolates by their capacity to produce different pock sizes on CAMs. The present study sought to demonstrate the relationship of pock size with the two antigenic types of HSV isolated from clinical samples of both genital and non-genital sites. Our study observed that almost all (92.9%) HSV isolates from genital lesions produced large pocks on the CAM, which were confirmed by the more reliable DFA method as HSV-2. Only one (7.1%) HSV isolate from a genital lesion produced small pocks and was confirmed by DFA test as HSV-1. On the other hand, all non-genital lesions produced small pocks and were confirmed by DFA test as HSV-1. A similar study from USA reported that most of the pocks produced by HSV-2 exceeded 1 mm in diameter at 3rd day¹². This was probably because the work was done with high passage laboratory strains rather than low passage clinical isolates, since repeated passage of HSV isolates tend to diminish the distinctive properties of the two types by means of selection and mutation. Another study from the UK which had adopted HSV isolates by egg passage first reported that the size of pocks produced by HSV-2 were 0.8–1 mm, while HSV-1 were 0.2-0.5mm¹⁸, which is comparable to our findings. Similarly, other studies to differentiate HSV isolates from clinical samples by pock size observed that pocks produced by HSV-2 isolates were comparatively larger (>0.5 mm) while HSV-1 were smaller (<0.5mm)^{15,17}.

Furthermore, our study observed that if CAMs are inspected at 5 days rather than at 3 days, larger lesions (2-3 mm in diameter) occurred with HSV-2 isolates, whereas, HSV-1 isolates remained typically small. Similar findings have been reported earlier¹⁴. This may be another significant determinant for the differentiation of HSV-1 and HSV-2.

Our study found the method of typing HSV isolates on CAM as very accurate. The technique employed for the study was relatively simple as eggs are readily available and also quite cheap. Moreover, inoculation on CAM is an easy procedure in comparison to other biological methods such as virus culture, and results can be obtained within 3 days. Therefore, in resource poor settings, the pock test on CAM of embryonated hen's egg may be used as a quick, easy, reliable and inexpensive biological marker to ascertain the antigenic types of HSV from clinical samples.

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