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A Sensitive Neutralization Assay for Influenza C Viruses Based on the Acetylesterase Activity HEF Glycoprotein

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ABSTRACT

Influenza C virus possesses specific neuraminate-O-acetylesterase as a receptor-destroying function. This enzymatic activity of the viral glycoprotein HEF (Hemagglutinin, esterase activity and fusion factor) can be visualized *in situ* by the use of distinct color substrates. Hereby the localization, as well as the quantity of synthesized HEF protein is detectable. We further developed the esterase staining technique for a rapid detection and typing of influenza C progeny virus and for sensitive quantification of low viral loads. Neutralizing antibodies, interfering with virus attachment, were utilized to determine the infectivity of the inoculum in an indirect manner. The amount of unneutralized, infectious virus could easily be quantitated by *in situ* staining of the HEF esterase activity in infected cells. An evaluation of infectivity is presented and is put into relation to hemagglutinating virus units. Both, virus and serum antibody titers can be reliably determined by the esterase-neutralization assay. In this study a serial dilution of human and different animals (swine, dog and rabbit) sera were tested by *in situ* estrase neutralization assay (ENA) and hemagglutination inhibition (HI) test. The results show that *in situ* ENAis a sensitive method for titration of infectious rate of virus and quantification of neutralizing antibody against influenza C virus in different sera. *Iran. Biomed. J. 5 (1): 27-32, 2001*

Keywords: Influenza C virus, Neutralization assay, *in situ* esterase activity, Virus titration.

INTRODUCTION

Biological, biochemical and genetic studies of influenza C viruses have been hampered by the lack of a suitable antibody neutralization and titration test. influenza C viruses are distinguished from influenza A and B viruses by immunological properties, the nature of the virion glycoprotein and the number of genomic segments [1, 2]. Influenza C viruses do not contain neuraminidase [3, 4] which is present in influenza A and B viruses. The surface protein of influenza C virus is a single type of glycoprotein, [5, 6] which has been termed HEF (Hemagglutinin, esterase activity and fusion factor)[7]. It displays three biological activities: (i) the receptor-binding activity, that mediates the attachment of the virus to N-acetyl-9-O-acetylneuraminic acid (Neu5, 9Ac$_2$) on glycoproteins or glycolipids of the cell surface [8], (ii) fusion with the host cell membrane [7] and (iii) the receptor-destroying activity, which is a neuraminate-O-acetylesterase [9, 10, 11]. The viral receptor-destroying enzyme hydrolyses acetic acid from the cellular receptor, thereby allowing release of the mature virus from infected cells [12, 13].

Wagaman *et al.* [13] have identified two substrate analogues, α-naphthyl acetate (ANA) and α-naphthyl propionate for the influenza C virus esterase. In the presence of pararosanilin, α-naphthyl acetate (ANA-P) serves as a substrate for the viral enzyme *in vitro* and can be employed to develop *in situ* esterase staining, specific for influenza C virus in Madine-Darby canine kidney (MDCK) cells. We modified this method and established a new titration and neutralization test for influenza C virus.

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MATERIALS AND METHODS

Virus. Strain Johannesburg/1/66 (C/JHB/1/66) of influenza C virus was used throughout this study. Stock virus was prepared by allantoic inoculation of 8-day-old embryonated hen eggs with 0.1 ml of an inoculum containing 2 HAU/ml. The allantoic fluid was harvested after incubation of the eggs at 33°C for 3 days, assayed by hemagglutination with chicken erythrocytes as described [14], and frozen at -70°C. The allantoic fluids used as a stock virus contained 256 HAU/ml.

Cell culture. Madine-Darby canine kidney (MDCK) cells were grown in Dulbecco´s medium containing 10% of FCS. Approximately 2×10^4 MDCK cells per 1 ml of medium were cultivated in 12-well tissue culture plates (Costar) at 37°C in a humidified atmosphere with 5% of CO_2.

Antisera. Human sera, for use in the neutralization test, were derived from diagnostic serum collection of Dept. of virology, "Institut fuer Medizinische Mikrobiology und Hygiene" Techn. University of Munich. Dog and swine sera were kindly provided by Prof. W. Kraft, from "I. Medizinische Tierklinik, LM-University of Munich." Rabbit antisera against influenza (C/JHB/1/66) virions were prepared by immunization of 4-month-old rabbits with two intravenous and two intramuscular injections of 0.5 ml of virus, containing 5000 HAU/ml at interval of two weeks. Withdrawal of blood was carried out 10 days after the last injection, on the 8th week. All sera were inactivated before use at 56°C for 30 min and stored at -20°C.

Hemagglutination inhibition (HI) test. Tests were carried out in U-shaped microtiter plates as described [15]. The sera were first mixed with chicken erythrocytes to absorb non-specific hemagglutinins. HI titration was then carried out in a microtiter plate against 4 HA units of the virus, 1% chicken erythrocytes and a phosphate saline (PBS, 0.01 M phosphate, 0.14 M NaCl, pH 7.2) as diluent. The results were read after the incubation at 4°C for 60 min, whereby the titer was defined as the reciprocal value of the last serum dilution where agglutination inhibition was clearly visible.

Preparation of esterase substrates. α-nahpthyl acetate (ANA) (Sigma Chemical Co., St. Louis, Mo) was prepared by the method of Li *et al*. [16] by dissolving ANA (10 mg) in ethylene glycol monomethyl ether (0.5 ml) and dilution to 10 ml with phosphate buffer (0.067 M, pH 6.3). Hexazonium pararosanilin (0.6 ml) was prepared as described [17]. Hexazotization of pararosanilin was made by mixing an equal volume of the pararosanilin solution and a fresh 4% sodium nitrite solution for 1 min. Befor use. The final pH of the incubating medium was adjusted with 1 N NaOH to pH 6.1 (range 5.8 to 6.5). The incubation medium was filtered before use. Both components ANA (10 ml) and Hexazonium pararosanilin (0.6ml) were mixed to obtain ANA-P, adjusted to pH 6.1 and filtered before use.

In situ esterase detection. The method of Wagaman *et al.* [13] was used to detect the esterase activity of influenza C virus in the infected MDCK monolayers. MDCK cells were grown in Dulbecco's MEM containing 10% (v/v) FCS. Prior to infection, monolayers were rinsed twice with PBS. After 1-hour adsorption at 33°C, using 250 µl of a stock influenza C viruses (128 HAU/ml), cells were washed again with PBS before adding Dulbecco's MEM containing 2% FCS. Flasks were incubated at 33°C for 24 h. After removal of the culture medium, monolayers were fixed for 30 s with cold buffered Formalin-acetone (1.4 mM Na_2HPO_4, 7.3 mM KH_2PO_4 , 45% acetone, 25% Formalin) and washed three times with distilled water. The fixed cells were incubated with 2 ml of ANA-pararosanilin (ANA-P) solution per well (25 mm^2) for 15 min at room temperature. After rinsing, infected cells were stained red under the light microscope.

Virus titration by estrase activity. Stock C/JHB /1/66 virus with a titer of 256 HAU/ml was diluted serially in PBS containing 2% of FCS. Then 0.1 ml from each dilution was added to the MDCK cell monolayers per well. After adsorption of virus for 1 h at 33°C the monolayers were again incubated at 33°C for 24 h and assayed for esterase activity.

Neutralization. Neutralization of virus infectivity was measured by the esterase neutralization assay using MDCK cell monolayers in 12-well plates. Serial dilutions (0.3ml) of human, dog and swine reconvalescence sera as well as immunized rabbit antisera against C/JHB /1/66 virus were reacted in Eppendorf tubes with 0.3 ml of virus that had been adjusted to a titer of 256 HAU/ml. For neutralization reaction, the virus-serum mixture was incubated for 1 h at room temperature under shaking. Then 0.2 ml of each mixture was inoculated with MDCK monolayers in two parallel setups. The monolayers were incubated at 33°C for 1 h. The virus-serum mixture was removed and the cells were rinsed with PBS. Then 1 ml MEM containing 2% of FCS was added to the monolayers per well and plates were incubated at 33°C for 24 h. Finally, monolayers were fixed and assayed for esterase activity. Human and animals sera, as listed above (HI-test positive), were used for positive controls and pre-immune rabbit serum and PBS served as negative controls.

RESULTS AND DISCUSSION

A new neutralization assay for influenza C virus has been developed on the basis of *in situ* esterase activity of the viral HEF glycoprotein. Our esterase-neutralization assay (ENA) is shown to produce reliable results in terms of specificity, sensitivity and reproducibility by application on the single cell level [18].

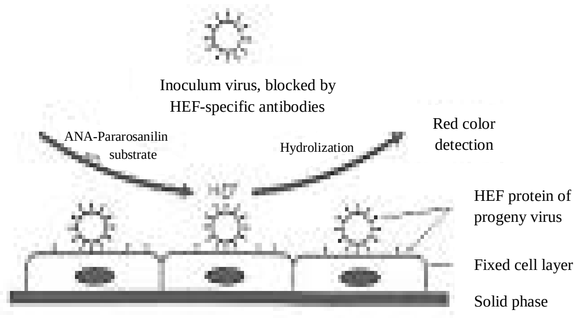

Fig. 1. Schematic representation of the esterase activity assay in the infected cell monolayers. The diagram represents hydrolization of ANA-P substrat by viral enzyme (HEF-esterase) and colour detection. Upon antibodiy neutralization, virus infectivity is blocked and is measured indirectly by the colour reaction produced by the progeny virus.

In principle, ANA the substrate analogue for the viral esterase in the presence of pararosanilin, is enzymatically hydrolized, resulting in a red *in situ* colour reaction (Fig. 1). This procedure allows the specific detection of influenza C virus HEF activity in the infected cells. Thus, specific neutralizing antibodies, blocking initial virus attachment, are set into proportion with the rate of infection and progeny production, which is subsequently measured in an indirect way by staining of the produced HEF esterase activity. On this basis, the titration of influenza C virus-reactive antisera can be utilized to determine their neutralizing end-points or, in other terms, to set a value for the rate of infectious virus.

In situ esterase staining was performed on infected MDCK monolayers using the described ANA-P substrate (Fig. 2). The color reaction was found to be highly specific for the viral enzymatic activity and was not detectable for cellular esterases in uninfected controls, or for monolayers infected with influenza A and B viruses (not shown). Red staining patterns were found in different cellular compartments or as complete surface reaction, consistent with the distribution of intracellular HEF expression [19]. The suitability of this *in situ* detection method for the quantification of virus samples used for infection was made by serial dilution studies of one representative C/JHB/1/66 specimen (Fig. 3). As presented, the virus titer directly correlates to the number of esterase-expressing cells, i.e. the infectivity rate of the inoculum can be measured by cell counting. Viral loads of an 500 HAU/ml-inoculum were used in dilutions between 1:64 and 1:32000 and these reacted within the range of determination.

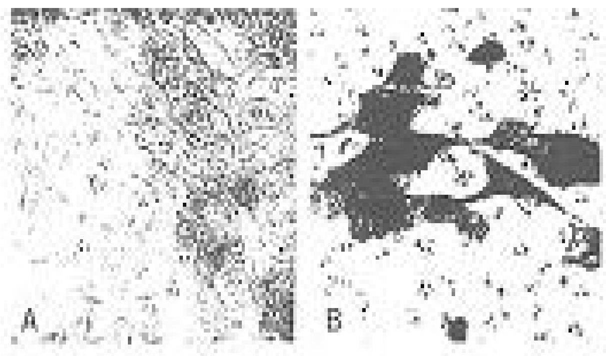

Fig. 2. *In situ* esterase activity in MDCK monolayers infected with stock C/JHB/1/66 virus. After 1 h adsorbtion at 33°C, the inoculum was removed, washed with cold PBS and incubated with MEM (supplemented with 2% of FCS). Monolayer were incubated at 33°C for 24 h, were fixed with cold buffer Formalin-acetone and reacted with ANA-P to show esterase activity. (A) Uninfected monolayer. (× 320), (B) infected monolayer with 0.2 ml inoculum per 4cm². (× 320).

The neutralizing effect of HEF-specific antibodies was demonstrated by serum titration in infection experiments (Table 1). For this, human and different animal sera with HI-titers between 1:512 (Human) and 1:160 (Swine) was serially diluted and subjected to a constant concentration of stock virus

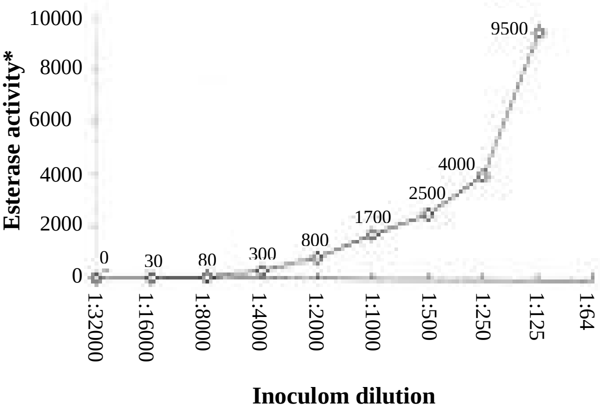

Fig. 3. Infectivity rate of influenza C virus, measured by *in situ* esterase activity in MDCK monolayers. Confluent MDCK monolayers were infected with 0.1 ml of stock C/JHB/1/66 virus (500/HAU/ml) of seriall dilutions between 1:64 and 1:32000. After 1 h adsorbtion at 33°C, the inoculum was removed, washed with cold PBS and incubated with MEM (supplemented with 2% FCS). Monolayers were incubated for 24 h, fixed with cold buffer formalin-acetone and incubated with ANA-P for detection of esterase activity. * The evaluation is given as stained cells per total number.

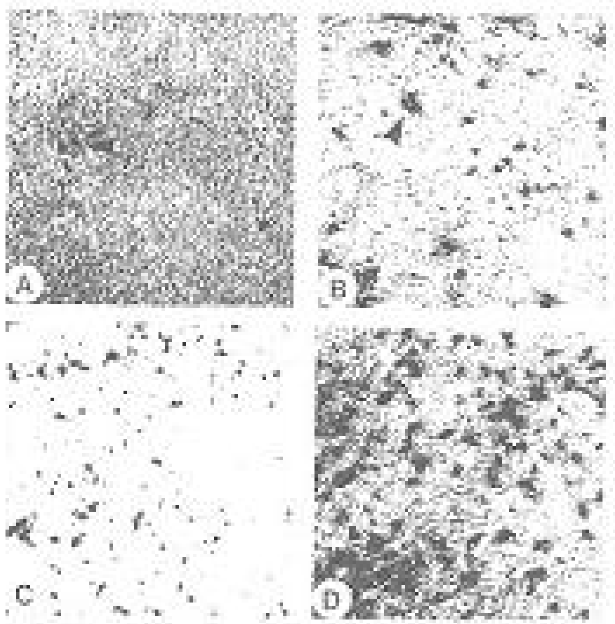

Fig. 4. Patterns of staining after serial infections according to the ENA procedure. Monolayers were incubated with mixtures of inoculum virus (125 HAU/ml) and the respective dilution of human serum. After 24 h, cells were harvested *in situ* stained and quantitated as shown. (A), HI titer of 1:32 (× 40); (B), HI titer of 1:16 (× 40); (C), HI titer of 1:8 (× 40); (D), HI titer of 1:4 (× 40).

C/JHB/1/66 (125 HAU/ml). The reactivity of the panel of antisera used was analyzed for its ability to neutralize the inoculum infectivity of influenza C virus by binding to structural virion epitopes. A clear effect in blocking virus replication was mirrored indirectly by the *in situ* esterase color development and was found for all antisera tested. The antisera with HI-titers > 1:32 were effective in neutralizing virus and inhibited esterase activity. Pre-immune control serum showed no significant inhibition. The neutralizing limit of each respective antiserum was depicted by cell counting. Titers of neutralization in this ENA measurement were attributed to HI-titers between 1:32 and 1:40. The cellular staining patterns of this experimental evaluation is given in Fig. 4. In dependence to the viral load, staining occurred in a patchy distribution over the monolayer. Note that an unquestionable distinction between virus-positive centers and RDE-free areas is already possible at low microscopic magnifications (× 40).

In conclusion, the establishment of a quantitative neutralization assay for influenza C virus satisfies the need for an alternative method to the critical limitations in plaque assays [20] or the classical HI test. Our technique was based on detection of productive virus replication by the staining of HEF-esterase activity [13]. While former neutralization tests evaluated the antibody-mediated inhibition on the antigen or infectivity level, for example in case of influenza viruses [21] or Mumps virus [22], this approach utilizes the indirect suppression of RDE function. Both, the infectious rates of uncharacterized virus samples and the quantitation of antibody titers in HEF-reactive sera, can easily be achieved by ENA. Further methodical improvements will concentrate on the very sensitive demands in ENA measurement handling material of low viral loads, for specific neutralization of a persistent influenza C virus variant [23] in cell culture, or as an alternative to the RT-PCR test for detection of influenza C in nosopharyngeal secretion [24] to distinguish type C virus from other respiratory viruses.

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Table 1. Comparison of esterase neutralization assay (ENA) with HI-titer[c]

Sera[a] Dilution Ĝ Sera +Virus[b] ĝ	**1:2**	**1:4**	**1:8**	**1:16**	**1:32**	**1:64**	**1:128**
Human	-	-	-	+[d]	++	+++	++++
	1:256	**1:128**	**1:64**	**1:32**	**1:16**	**1:8**	**1:4**
Dog	-	-	-	+	+++	++++	++++
	1:160	**1:80**	**1:40**	**1:20**	**1:10**	**1:5**	**1:2.5**
Swine	-	-	+	++	+++	++++	++++
	1:80	**1:40**	**1:20**	**1:10**	**1:5**	**1:2.5**	**1:2.5**
Rabbit	-	-	-	-	+	++	+++
	1:256	**1:128**	**1:64**	**1:32**	**1:16**	**1:8**	**1:4**
Rabbit	-	-	-	-	-	-	-
(Without virus)	**1:256**	**1:128**	**1:64**	**1:32**	**1:16**	**1:8**	**1:4**
Pre-immune	++	+++	++++	++++	++++	++++	++++
Rabbit serum	-	-	-	-	-	-	-
PBS + Virus	++	+++	++++	++++	++++	++++	++++
	-	-	-	-	-	-	-

[a] Serially dilution of diferent animals and human sera; [b] Influenza C/JHB/1/66 with 125 HAU/ml; [c] Antibody titer of the sera versus influenza C virus were determined by hemagglutination inhibition test. All sera with HI-titer>1:32 neutralized virus infectivity and inhibited esterase activity; [d] Rate of esterase neutralization assay per well: +, stained cells between 1 and 50 per well; ++, stained cells between 51 and 500 per well; +++, stained cells between 501 and 1000 per well; ++++, stained cells more than 1000 per well.

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