Influence of the Temperature and Cell Line on the Adsorption and Replication of Five Fish Rotaviruses

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The infectivity of five fish rotaviruses was assayed on 4 fish and two mammalian cell lines at different temperatures. These viruses were isolated recently from turbot (TRV), smelt (SRV), striped bass (SBR) and Atlantic salmon (ASR and HBR). The two mammalian lines, MA 104 and MDBK, were not susceptible to any of the viruses assayed, although viruses were adsorbed by the cells at certain temperatures. Of the four fish cell lines employed (CHSE-214, FHM, EPC and BB), only FHM cell failed to support the growth of all the viruses. The EPC cells were susceptible, but the titers were low ($\leq 10^{5.5}$ TCID₅₀/ml). Although the BB cells proved to be very useful for the replication of SRV, the Atlantic salmon rotavirus failed to grow in this cell line. The CHSE-214 cell is the optimal cell line for all these rotaviruses because they produced relatively high titers (between $10^{6.25}$ and $10^{9.25}$ TCID₅₀/ml) when the cells were incubated at 15°C.

One of the more recent groups incorporated to the fish virology is the family Reoviridae, being reported the first isolates belonging to this family at the end of the 70's (Meyers, 1979; Plumb *et al.*, 1979), and since then many other researchers have reported the isolation of reo-like viruses. Although these viruses share properties with Reoviridae, they show characteristics not exactly coincident with any of the genera included in this family. Therefore, Winton *et al.* (1987) suggested the name Aqua-reovirus for a new genus to group these viruses isolated from poikilothermic animals. Recently, new rotaviruses have been reported from various fishes (Baya *et al.*, 1990; Dopazo *et al.*, 1989; Hetrick *et al.*, 1989; Lupiani *et al.*, 1989; Samal *et al.*, 1991).

As is well known, one of the first steps to characterize a new virus is the study of its

infectivity on different cell lines, as well as its thermostability. Little information, however, is available on these matters of the aquareoviruses (Amend *et al.*, 1984; Hsu *et al.*, 1989; Meyers, 1979; Plumb *et al.*, 1979; Schwedler and Plumb, 1982).

In this paper we compare the adsorption ability and the infectivity of 5 recent rotavirus isolates on 4 fish and 2 mammalian cell lines. In addition, the influence of the temperature on the adsorption and replication of these viruses was also evaluated.

Material and Methods

Viral Strains

The five fish rotaviruses used in this study were turbot rotavirus (VTR) isolated in Spain from diseased turbot (*Scophthalmus maximus*) by Lupiani *et al.* (1989); smelt rotavirus (SRV), isolated in Canada from smelt (*Osmerus mordax*) by Moore *et al.* (1988) and identified by Marshall *et al.* (1990); striped bass rotavirus (SBR), isolated from diseased striped bass (*Morone sax-*

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atilis) by Baya *et al.* (1990) in USA; ASR and HBR, isolated from Atlantic salmon (*Salmo salar*) by Moore in Canada, and Hetrick and Baya in USA, respectively (unpublished isolations).

Cell Lines and Culture Conditions

The fish cell lines assayed were CHSE-214, EPC, FHM and BB. The cells were continuously cultured in Eagle's minimum essential medium (EMEM), supplemented with 10% foetal calf serum (FBS), penicillin (100 iu/ml) and streptomycin (100 µg/ml). EPC, BB and FHM cells were incubated at 25°C, and CHSE-214 cells at 15°C. Monolayers were maintained at 15°C using EMEM with no serum.

Two mammalian lines, MA104 (fetal rhesus monkey) and MDBK (bovine kidney), commonly used for the culture of rotavirus from homeotherms, were also assayed. They were cultured at 37°C with EMEM supplemented with 6% FBS, and for the maintenance EMEM without serum was used.

Viral Adsorption on Cells

The six cell lines were cultured in 25 cm² flasks. When the monolayers were confluent, the medium was withdrawn. Then, 1 ml of the corresponding virus was inoculated into the flasks at a multiplicity of infection (M.O.I.) ranging between 0.01 and 0.1. Flasks of the mammalian cells were incubated at 15, 20, 25, 30 and 37°C. Whereas the adsorption assays on CHSE-214, FHM and CPC cells were conducted at 15, 20 and 25°C, the BB cells were incubated also at 30°C. After an adsorption period of 1 h, each inoculum was totally recovered and titrated to determine the quantity of virus that did not adsorb to the cells.

Titration was carried out in 96 well plates of CHSE-214 cells and the titers were calculated according to Reed and Muench (1938). The results were expressed as percentage of virus adsorbed, with respect to the total virus inoculated.

Cell Susceptibility

After the adsorption period, 5 ml of EMEM without serum were added to the inoculated flasks and then incubated at the corresponding

temperature. Flasks were observed daily to determine the time to appearance of CPE and the time to total destruction of the monolayer. In the case of FHM and EPC cells, the cell susceptibility to the rotavirus was also tested at 30°C. Control flasks of each cell line were incubated at the different temperatures. When the monolayers were totally destroyed, the flasks were maintained at -80°C until viral titration. After a maximum incubation period of 45 days, flasks with no CPE were rejected. When CPE was observed but not total destruction occurred, the cells were disrupted by 2 rapid cycles of freezing and thawing, and the cell suspension was frozen at -80°C until titration. Virus titers were calculated according to the Reed & Muench method using 96 well plates of the respective cell lines.

Temperature Sensitivity Assay

This assay was conducted to determine the possible viral inactivation occurring during the adsorption period. The five fish viruses were diluted in EMEM to obtain an initial titer of about 10⁵ TCID₅₀/ml. The dilution of each virus was distributed in 4 ml aliquots, using 5 ml screw-cap glass vials. Each vial was incubated at 10, 15, 20, 25, 30 and 37°C, respectively. Samples of 0.1 ml were taken at 0 and 1 h, and immediately titrated.

Results

Viral Adsorption on Cells

The percentages of virus adsorbed on the six cell lines at the temperatures tested are shown in Fig. 1. We did not observe a clear relationship between temperature and the adsorption capability of the viruses. As shown in the figure, in CHSE-214 cells at 20°C all the viruses adsorbed in percentages higher than 80%, and in FHM cells at 25°C. On EPC and BB cells we did not find a temperature at which all the viruses exhibited a high adsorption ability (we subjectively considered percentages of adsorption higher than 75% as high adsorption ability). Even HBR showed low percentages of adsorption at all the temperatures assayed in these two cell lines, and in BB it was unable to adsorb at 20, 25 or 30°C.

Fig. 1. Adsorption of the five fish rotaviruses on cell lines.

Data are shown as percentage of virus adsorbed on the cells.

TRV, □; ASR, ▨; HBR, ▩; SBR, ■; SVR, ▤.

*: Not Tested.

The mammalian cell lines showed the highest variability. SBR and SRV showed high percentages of adsorption to both cell types (mammalian and poikilothermic). In contrast, ASR and HBR showed low adsorption capability on these cells.

To determine the optimum temperature for the incubation of the five viruses during the adsorption period, we analyzed the percentage of adsorption of these viruses on each cell line and at each temperature, by means of the mean (μ) and the standard deviation (s). In this sense, a high μ of adsorption percentages with a low s indicates that the five viruses present a high adsorption ability on the corresponding cell line and incubation temperature. On the other hand, the higher s value indicates the higher differences in adsorption ability between the five viruses. As shown in Table 1, high average of percentages of adsorption with low s were obtained in two cases, viz. on CHSE-214

Table 1. Effect of temperature on the adsorption of the five fish rotaviruses.

Cell line	Temperature (°C)				
		15	20	25	30 37
CHSE-214	μ	71.8	91.6	79.4	
	s	17.55	6.02	20.59	
FHM	μ	71.4	85.8	90.0	
	s	22.87	11.55	5.06	
EPC	μ	78.0	79.8	84.8	
	s	19.56	21.09	13.76	
BB	μ	65.6	66.8	72.0	62.0
	s	18.99	34.56	36.27	35.71
MA104	μ	64.7	64.0	77.6	65.0 56.6
	s	45.73	37.41	19.97	45.96 37.06
MDBK	μ	64.2	47.8	53.8	44.8 69.3
	s	37.25	43.65	44.18	40.25 40.18

μ : mean of the adsorption of the viruses on each cell line.

s : standard deviation of the mean.

cell line at 20°C and on FHM cell line at 25°C. This result indicates that in these two lines the use of the same incubation temperature could be possible for an effective adsorption of the five viruses. In the rest of the cell lines the minimum *s* value was 13.75, which means that in these cell lines the temperature affects the adsorption ability of the five fish rotaviruses differently.

Cell Susceptibility and Virus Replication

Only three cell lines (CHSE-214, EPC and BB) were able to support the replication of the viruses, EPC and BB at 15°C, and CHSE-214 at 15 and 20°C (Table 2). The five rotaviruses grew fastest (in less than 1 week) in CHSE-214 line, causing destruction of the monolayer in one to two weeks. BB was the optimum for SBR and SRV which produced CPE in 4 days and monolayer destruction in 1–2 weeks, but this line was not susceptible for ASR. The replication of all the viruses was very slow in EPC cell line and, in the case of TRV and SRV no destruction of the monolayer was detected.

The results were quite similar when testing the titers of virus produced in these cells since there is a certain relationship between the time to appearance of CPE and the viral titer ob-

Table 2. Cell susceptibility to the five fish rotaviruses.

Cell line	Temperature (°C)	Rotaviruses				
		TRV	ASR	HBR	SBR	SRV
CHSE-214	15	5/16*	3/14	3/12	2/6	3/13
	20	4/20	3/15	2/7	3/13	3/15
	25	NC	NC	NC	NC	NC
EPC	15	30/—	29/33	30/40	30/38	35/—
	20	NC	NC	NC	NC	NC
	25	NC	NC	NC	NC	NC
	30	NC	NC	NC	NC	NC
BB	15	30/—	NC	20/—	4/16	4/5
	20	NC	NC	NC	NC	NC
	25	NC	NC	NC	NC	NC
	30	NC	NC	NC	NC	NC

(Data are shown in days post infection).

*: Days to appearance of CPE/Days to total destruction.

—: No total destruction detected after 45 days.

NC: No CPE detected after 45 days.

Fig. 2. Replication of fish rotaviruses on cell lines. TRV, □; ASR, ▨; HBR, ▩; SBR, ▪; SRV, ▫.

tained (the faster development of CPE and total lysis corresponded to the higher viral titer obtained). As shown in Fig. 2, CHSE-214 cells again proved to be the optimum for rotavirus production, except for SRV, which showed the highest titer in BB cells at 15°C ($10^{9.25}$ TCID₅₀/ml). In general, the titers produced in CHSE-214 at 15°C were greater than at 20°C, with the maximum virus titer obtained being $10^{9.25}$ TCID₅₀/ml for SBR.

EPC cells showed the lowest susceptibility to the five fish rotaviruses, displaying a maximum titer of $10^{5.5}$ TCID₅₀/ml for SBR. BB exhibited a variable behaviour. Whereas SRV and SBR grew to high titers in BB, this line had low susceptibility to HBR ($10^{4.5}$ TCID₅₀/ml) and TRV ($10^{1.75}$ TCID₅₀/ml), and the ASR failed to grow in this cell line.

Thermal Inactivation

The possible loss of infectivity during the 1 h adsorption period at different temperatures was followed. No significant decrease in infectivity of the five rotaviruses was seen at any temperature, the losses being never higher than 0.33 log, which rules out possible thermal inactivation of the rotaviruses during the adsorption period.

Relation between Adsorption and Viral Replication

In those cases when the cells exhibited susceptibility to the fish rotaviruses, we compared

Fig. 3. Relationship between adsorption and replication of fish rotaviruses on cell lines.

Histograms show the percentages of adsorption of the viruses on the cell lines; the scale is shown at left vertical axis. (TRV, □; ASR, ▨; HBR, ▩; SBR, ▤; SRV, ▦).

The linegrams show the viral production in Log of the TCID₅₀/ml; the scale is shown at right vertical axis. A, on CHSE at 15°C; B, on CHSE at 20°C; C, on EPC at 15°C; D, on BB at 15°C.

Correlation coefficients: A, $CC = -0.38$ (no significant, NS); B, $CC = 0.572$ (NS); C, $CC = -0.476$ (NS); D, $CC = 0.984$ (Significant, $p < 0.01$).

the percentages of viral adsorption and the viral titers of the five viruses, to know if there was some relationship between adsorption and replication. We employed a correlation test, with the null hypothesis being $H_0: \rho = 0$ (this implies that both variables are uncorrelated) (Sokal and Rohlf, 1981). If the correlation coefficient (CC) is significant, both variables analyzed are correlated.

As shown in Fig. 3, only in BB cells we can reject H_0 ($CC = 0.984$, $p < 0.01$), which means that a correlation exists between adsorption and replication, and then, those viruses with higher adsorption ability will show higher replication titers in this cell line at 15°C. In the other cases, no correlation exists between both parameters.

Discussion

In spite of the development of highly sensitive methods for the rapid detection of fish viruses such as immuno-dot or ELISA techniques, cell cultures are still necessary for the isolation,

production and characterization of a virus. For this reason, it is important to determine the optimal conditions for growing virus, including the cell line to be employed and the optimum temperature of incubation for its replication. We agree with Fendrick *et al.* (1982), that it is important to take into account not only the rapidity of CPE appearance but also the final viral titer obtained.

In the present study, only three cells (CHSE-214, EPC and BB) of the six cell lines tested supported the replication of the five fish rotaviruses (Fig. 2). As observed, SRV produced the highest titer in BB, while the other 4 viruses replicated better in CHSE-214. Regardless of the cell lines, the optimum temperature for replication of these viruses was 15°C, and they replicated at 20°C only in the CHSE-214 cells.

As related above, the five rotaviruses failed to produce CPE in FHM cells. This result agreed with those reported previously by the authors for the TRV (Lupiani *et al.*, 1989). Some other researchers have published similar behaviour of this cell line for other reo- or

rota-like fish viruses, as the 13P₂ virus from oyster, *Crassostrea virginica* (Meyers, 1979), the catfish reovirus (Amend *et al.*, 1984) or the landlocked salmon virus (Hsu *et al.*, 1989). However, other authors (Ahne and Kölbl, 1987; Plumb *et al.*, 1979; Schwedler and Plumb, 1982) found that the FHM cells were useful to culture golden shinner and chum salmon reoviruses.

Our fish rotaviruses did not grow in the two mammalian cell lines (MA104 and MDBK) generally used for the culture of homeothermic rotaviruses, which supports their poikilothermic origin. This finding is in agreement with the distinct protein and RNA electrophoretic patterns exhibited by the fish rotaviruses in comparison with those shown by SA11 (bovine rotavirus) and Reo type I (human reovirus) viruses (Samal *et al.*, 1989; Samal *et al.*, 1991).

Interestingly, when the adsorption assays were conducted, most of the viruses were able to adsorb on all the cell lines, at least at some temperatures. However, the HBR virus exhibited a narrow spectrum of adsorption because the virus adsorbed on BB and MA104 cells only at one temperature, and it failed to adsorb on MDBK at any temperature tested.

A clear relationship was not found between the adsorption ability and the temperature of incubation during the viral adsorption. So, the increase of temperature do not always affects increasing or decreasing the adsorption of the virus. We could assume that the viruses were inactivated during the adsorption period mainly by the highest temperature. In this way, the titer of virus adsorbed (measured as the difference between the total virus inoculated and the titer of virus recovered after that period) would be higher than the real value. However, this possibility was ruled out because in the thermostability assay the rotaviruses proved to be very stable at all the temperatures tested.

The aim of this study was to determine which cell line and which temperature of incubation was the optimal for the replication of these viruses. From the results obtained we conclude that CHSE-214 can be considered as the optimal cell line for the culture of the rotaviruses because they produce relatively high titers when the infected cells are incubated at 15°C.

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