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Assessment of commercial test kits for identification of spring viraemia of carp virus

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ABSTRACT: Two test kits for the identification of spring viraemia of carp virus (SVCV), one an enzyme-linked immunosorbent assay (ELISA) using a rabbit polyclonal antiserum, and the other an indirect fluorescent antibody test (IFAT) using a mouse monoclonal antibody, were assessed for specificity using a range of virus isolates. The test viruses were selected from 4 recently described genogroups of piscine rhabdoviruses: Genogroup I (SVCV), Genogroup II (grass carp rhabdovirus), Genogroup III (pike fry rhabdovirus) and Genogroup IV ('tench rhabdovirus'). The test viruses included SVCV isolates from all 4 subgroups of Genogroup I. The ELISA was non-specific for these viruses and did not distinguish between SVCV and isolates from the other 3 Genogroups. However, the IFAT was too specific and detected SVCV isolates from only 1 of the 4 SVCV subgroups. Reliance on these test kits alone could result in misidentification of this OIE notifiable disease.

KEY WORDS: SVCV · IFAT · ELISA · Serological comparison · Identification

INTRODUCTION

Spring viraemia of carp (SVC) is a serious haemorrhagic disease of carp and certain other fish species, caused by SVC virus (SVCV), a rhabdovirus tentatively classified in the genus *Vesiculovirus* (Ahne et al. 2002). SVC is a disease notifiable to the World Organisation for Animal Health (OIE) (OIE 2003). The method recommended by the OIE for screening fish is isolation of the virus in cell culture followed by the indirect fluorescent antibody test (IFAT) or enzyme-linked immunosorbent assay (ELISA) as rapid presumptive identification methods. Both of these methods can also be used to detect virus antigen directly in tissues of clinically infected fish. However, serological methods for identification of SVCV are not straightforward, as some other vesiculoviruses affecting fish that are not notifiable to the OIE, such as pike fry rhabdovirus (PFR), cross-react with antisera against SVCV (Clerx & Horzinek 1978, Clerx et al. 1978, Jørgensen et al. 1989, Ahne et al. 1998). SVCV may be distinguished from those other rhabdoviruses using the ELISA by undertaking reciprocal tests using antisera produced against SVCV and one of the heterologous viruses, then comparing the degree of reaction of the homologous and heterologous viruses with both antisera (Rowley et al. 2001, Way et al. 2003), but such ratios cannot easily be used in the IFAT.

Four Genogroups of piscine vesiculo-like viruses have recently been identified based on partial G-gene sequence analysis (Stone et al. 2003): Genogroup I comprises SVCV isolates, Genogroup II comprises a single isolate from the grass carp, Genogroup III comprises PFR and Genogroup IV comprises isolates mainly from cyprinids, but also includes salmonid isolates, and isolates previously identified as PFR by serology. Genogroup IV has provisionally been termed the tench rhabdovirus group after the oldest virus isolate allocated to it (tench isolate; Ahne et al. 1982). Genogroup I (SVCV) was further divided into 4 subgroups: a, b, c and d. The isolates in the different Genogroups had a high level of nucleotide sequence diversity, but also a high level of amino acid sequence identity, which was suggested as an explanation for the high degree of serological cross-reaction between isolates from different genogroups. Commercially available test kits have been produced for the serological identification of SVCV, and we report here on their specificity against isolates from Genogroups I, II, III and IV.

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MATERIALS AND METHODS

Viruses and propagation. Viruses were selected from distinct branches of the neighbour-joining distance tree (Table 1) within a Genogroup or subgroup (Stone et al. 2003, Dikkeboom et al. 2004, D. M. Stone pers. comm.) and grown at 20°C in *Epithelioma papulosum cyprini* cells (Fijan et al. 1983) or fathead minnow cells (ATCC CCL 42) (isolate WI02-131 only) as described previously (Hill et al. 1975, Way 1991), and harvested when complete cytopathic effect (CPE) was observed. The viruses were titrated in 96-well cell cultivation plates and $TCID_{50}$ values were calculated according to Kärber (1931). For the IFAT, virus-infected cell culture harvests were diluted to 1:100 and 1:1000 in cell culture medium and used to inoculate the appropriate cell cultures on 24-well IWAKI plates (Scientific Laboratory Supplies). Following incubation for 16 to 20 h at 20°C, the medium was removed, and the cells were fixed with the solution provided with the test kit, or with 80% acetone for the in-house IFAT (see below). For the ELISA, virus-infected cell culture harvests were diluted in diluent supplied with the kit. The remaining steps of the ELISA and IFAT were conducted according to the procedures supplied with the kits; cells inoculated with both the 1:100 and 1:1000 dilutions of virus were tested in the IFAT. An in-house rabbit polyclonal antiserum (produced against SVCV isolate S30) was used as a positive control for the IFAT.

Test kits. Kit SVC048, which is an ELISA using a rabbit polyclonal antiserum was obtained from TestLine, (Brno, Czech Republic) and Kit BIO K12, which is an IFAT using a mouse monoclonal antibody (MAb) came from Bio-X Diagnostics SPRL (Jemelle, Belgium). Tests were conducted according to the procedures specified by the manufacturers with the following exceptions. TestLine specified the use of doubling dilutions of sample from 1:2 to 1:256, but doubling dilutions from 1:20 to 1:2560 were used initially so that the results could be compared with those previously obtained from an in-house ELISA (Way 1991, C. B. Longshaw unpubl. data); in cases where the 1:20 dilution was negative, the test was repeated using doubling dilutions starting at 1:2. Bio-X suggests that the sample should be a fish extract potentially containing virus, and the cells should be fixed when CPE is observed, whereas the samples used here were cell culture harvests, and the cells were fixed after overnight incubation.

Table 1. Virus isolates used to assess the specificity of the TestLine and Bio-X kits for identification of spring viraemia of carp virus (SVCV)

Group[a]	Isolate	Host and geographic origin	Source/Donor
Ia	E232	Isolated in England from koi carp *Cyprinus carpio*, but with a connection to Asia	This laboratory
	980528 1.1	Isolated in England from koi carp imported from China	This laboratory
	D120	Isolated in England from koi carp, but with a connection to Asia	This laboratory
	NC02-46	Koi carp, USA	Goodwin (2002), Dikkeboom et al. (2004)
	WI02-131	Common carp *C. carpio*, USA	Dikkeboom et al. (2004)
Ib	E134	Common carp, illegally imported into England	This laboratory
	RHV	Rainbow trout *Oncorhynchus mykiss*, Ukraine	I. Shchelkunov, Russia
	2/90	Common carp, Moldova	I. Shchelkunov, Russia
Ic	NI-5	Bighead carp *Aristichthys nobilis*, Ukraine	I. Shchelkunov, Russia
	P4	Common carp, Russia	I. Shchelkunov, Russia
Id	770346	Common carp, England	This laboratory
	880163	Common carp, England	This laboratory
	940626 1.10	Golden tench *Tinca tinca*, England	This laboratory
	950255 3.1	Rudd *Scardinius erythrophthalmus*, England	This laboratory
	M2-78	Silver carp *Hypophthalmichthys molitrix*, Moldova	Shchelkunov & Shchelkunova (1989)
	N3-14	Grass carp *Ctenopharyngodon idella*, Ukraine	I. Shchelkunov, Russia
	S30	Common carp, Yugoslavia	Fijan et al. (1971)
II	V76	Grass carp, Germany	Ahne (1975)
III	PFR F4	Pike *Esox lucius*, France	de Kinkelin et al. (1973)
IV	994663 1.1	Bream *Abramis brama*, England	This laboratory
	S64	Tench *T. tinca*, Germany	Ahne et al. (1982)
	950237	Tench, England	This laboratory
	994602 3.1	Crucian carp *Carassius carassius*, England	This laboratory
	84-4	Brown trout *Salmo trutta*, Northern Ireland	Adair & McLoughlin (1986)

[a]Genogroup and subgroup according to Stone et al. (2003)

RESULTS AND DISCUSSION

The results of this comparative study are summarised in Table 2. Virus titres ranged from $10^{7.125}$ to $10^{9.25}$ $TCID_{50}$ ml^{-1} and no attempt was made to adjust the viruses to the same titre prior to the ELISA. Although there may have been different amounts of virus antigen in the preparations, the $TCID_{50}$ titre data acted as a check that isolates which did not react to a high titre in the ELISA had a titre of infectious virus comparable with isolates that did react to a high titre. For instance, isolate 84-4 had the lowest ELISA titre at 1:8, but had a slightly higher $TCID_{50}$ titre than isolate E232, which had an ELISA titre of 1:40, and consequently the low ELISA titre was unlikely to be the result of a low amount of infectious virus. All the isolates tested were positive in the ELISA, including the 7 isolates that were not genetically SVCV, which was mirrored by the IFAT results using the in-house polyclonal antibody. The range of ELISA titres across all genogroups was 1:8–1:160 and the most frequent titre was 1:40. Genogroup 1 (SVCV) isolates had ELISA titres ranging from 1:16–1:160, and the most frequent titre obtained was 1:40. There did not appear to be any relationship between $TCID_{50}$ titre and ELISA titre, which reflects the fact that different virus harvests are likely to have different levels of total antigen. For that reason we did not attempt to relate sensitivity of the ELISA to titre of infectious virus; however, the manufacturer of the test kit claims a sensitivity of $10^{2.8}$ to $10^{3.5}$ $TCID_{50}$ per 0.1 ml examined. The SVCV isolates did not all have high ELISA titres: 3 had a titre of 1:16 and 2 a titre of 1:20, which were equivalent to, or lower than, the majority of the non-SVCV isolates.

The MAb-based IFAT was more specific, but it only reacted with isolates in SVCV Subgroup Id, i.e. isolates originating mainly from central and western Europe. However, isolate M2-78 from Subgroup Id was not detected by the MAb. To further investigate that finding, 2 more isolates from Subgroup Id, 880163 and N3-14, that were the nearest neighbours to isolate M2-78 on the same branch of the neighbour-joining distance tree (Dikkeboom et al. 2004, D.M. Stone pers. comm.) were tested using both kits. Isolate 880163 had a titre of infectious virus of $10^{7.67}$ $TCID_{50}$ ml^{-1}, was detected by the ELISA at a dilution of 1:16, and by both the in-house IFAT and the IFAT test kit. Isolate N3-14 had a titre of infectious virus of $10^{7.5}$ $TCID_{50}$ ml^{-1}, was detected by the ELISA at a dilution of 1:16 and by the in-house IFAT, but was not detected by the IFAT test kit. Isolate N3-14 was closer than isolate 880163 to isolate M2-78 on the neighbour-joining distance tree. Isolates N3-14 and M2-78 were also isolated from neighbouring countries (Moldova and Ukraine), whereas 880163 was isolated in England. The instructions for use of the IFAT test kit describe the starting inoculum for the cell cultures as a fish tissue homogenate and state that the test should be performed when CPE is observed. However, in this study the inoculum was virus that had already been isolated, and the test performed before significant CPE (if any) was observed. In our experience, when cells are inoculated with a virus isolate and fixed when CPE is observed, the majority of the cells will fluoresce. Whereas, if they are fixed before CPE is observed, plaques of fluorescence will be observed; this provides a good contrast between infected and non-infected cells. Our use of previously isolated virus and fixation before CPE was observed is unlikely to have affected the results obtained with the

Table 2. Detection of selected fish vesiculoviruses using the TestLine ELISA and Bio-X IFAT kits for the identification of spring viraemia of carp virus (SVCV). +: positive fluorescence; –: no fluorescence

Group	Isolate	$TCID_{50}$ ml^{-1}	ELISA titre (reciprocal)[a]	IFAT (Bio-X)[b]	IFAT (in-house)[b]
Ia	E232	$10^{7.5}$	40	–	+
	980528 1.1	$10^{8.25}$	40	–	+
	D120	$10^{8.625}$	40	–	+
	NC02-46	$10^{8.625}$	40	–	+
	WI02-131	$10^{8.5}$	160	–	+
Ib	E134	10^{8}	80	–	+
	RHV	$10^{8.25}$	40	–	+
	2/90	$10^{7.125}$	16	–	+
Ic	NI-5	$10^{8.5}$	80	–	+
	P4	$10^{8.5}$	80	–	+
Id	770346	$10^{8.75}$	40	+	+
	940626 1.10	$10^{8.25}$	20	+	+
	950255 3.1	$10^{8.375}$	64[c]	+	+
	M2-78	$10^{7.75}$	20	–	+
	S30	10^{8}	40	+	+
II	V76	$10^{7.875}$	16	–	+
III	PFR F4	$10^{9.25}$	80	–	+
IV	994663 1.1	$10^{8.625}$	40	–	+
	S64	$10^{8.625}$	40	–	+
	950237	10^{9}	40	–	+
	994602 3.1	$10^{8.75}$	20	–	+
	84-4	$10^{7.75}$	8	–	+

[a]Titre determined as the last sample dilution giving a mean absorbance value > 0.1
[b]Cells were inoculated at 1:100 and 1:1000 dilutions and fixed 16 to 20 h later. The dilution used to inoculate the cells made no difference to the results
[c]This isolate was retested using doubling dilutions

IFAT kit because: (1) positive test results were obtained for some SVCV isolates with the IFAT kit, (2) the information supplied with the kit indicated that positive results can occur in the absence of CPE, and (3) all cell cultures inoculated with virus had been incubated for a sufficient amount of time to give positive results with the in-house IFAT which used a polyclonal antiserum. Our conclusion is that the MAb recognises epitopes that are present on some, but not all, SVCV isolates. Isolates from SVCV Subgroup Ia (isolates from the USA or with an Asian connection) were only weakly neutralised by in-house polyclonal antisera produced against SVCV from Subgroup Id (Dikkeboom et al. 2004, K. Way pers. comm.), but isolates from Subgroups Ib and Ic are neutralised to a similar extent as isolates from Subgroup Id using the same antisera (K. Way pers. comm.). Those observations suggest that Subgroup Ia isolates share few common neutralising epitopes with Subgroup Id isolates. The Bio-X IFAT results also suggest that there may be serological differences between SVCV isolates from different geographic areas.

A MAb-based IFAT kit from an unnamed manufacturer has been previously tested for its specificity against SVCV and PFR (Ariel & Olesen 2001) using a small number of isolates. The MAb in that kit detected all 4 SVCV isolates tested, and 1 of 3 isolates designated as PFR. The kit did not detect isolate V76 (originally designated as PFR, but nucleotide analysis has shown it to be the sole member of Genogroup II; Stone et al. 2003), nor isolate S64 (originally designated as PFR, but nucleotide analysis has allocated it to Genogroup IV; Stone et al. 2003). This is in agreement with our results. The reference PFR used by Ariel & Olesen (2001) was detected by the kit they tested, but the reference PFR (from the same donor) used in this study was not detected by the Bio-X IFAT kit. That may be because the MAbs in the kits assessed in both studies had different specificities, or because the 'reference' PFR used by both laboratories was not in fact the same virus. The PFR used in this study was confirmed as being genetically different from SVCV isolates (Stone et al. 2003), and is the sole member of Genogroup III.

It is clear that accurate serological identification of these vesiculoviruses is very difficult with the polyclonal and monoclonal antibodies currently available either from commercial sources or research laboratories. The serological identification of 'PFR' is particularly so. Rhabdoviruses isolated from roach (Haenen & Davidse 1989), brown trout (Adair & McLoughlin 1986), false harlequin (Ahne & Thomsen 1986), tench (Ahne et al. 1982) and grass carp (Ahne 1975) originally were described as PFR based on neutralisation data, but all have been shown by nucleotide sequence analysis to be different from the reference PFR (Stone et al. 2003). All are members of Genogroup IV, except the grass carp isolate, which is the sole member of Genogroup II. Likewise rhabdovirus isolates from bream, roach, brown trout and rainbow trout in Northern Ireland (Rowley et al. 2001) and tench, crucian carp, bream, roach, grass carp and chub in England (Way et al. 2003) appeared to be isolates of PFR by the neutralisation test. However, in ELISA comparisons the isolates appeared to be neither PFR nor SVCV, and 'intermediate' between the 2. Nucleotide sequence analyses showed that the isolates were all members of Genogroup IV.

The TestLine ELISA kit assessed here could be used as a preliminary screen of diseased fish, or of cell culture supernatants from diagnostic tests, but a positive result would only show that the virus belonged to one of the 4 currently recognised genogroups of fish vesiculoviruses. Further analyses would need to be done, such as RT-PCR followed by nucleotide sequence analysis for more complete identification. However, the latter technique is expensive to use on a large number of samples, and is not available to every laboratory. In order to have a standardised, accurate way of identifying and distinguishing SVCV from serologically related vesiculoviruses, this laboratory has developed a method using reverse hybridisation (A. M. Sheppard et al. unpubl.).

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