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Attempts to Serologically Classify Feline Caliciviruses on a National and an International Basis

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A serological classification of five feline calicivirus (FCV) strains of Swiss origin and 13 isolates of Austrian origin was attempted. The antisera used had been prepared in rabbits against the five Swiss strains and in goats against six American strains. Homologous and heterologous neutralization tests were made in tube cultures using sera at dilutions that contained 20 antibody units, in unabsorbed sera at low dilution, and in sera after subjection to three consecutive absorptions with lyophilized feline liver powder. None of these procedures resulted in the delineation of serotypes. A study using 20 antibody units yielded FCV strains with broad-spectrum antigenicity, which seem promising for vaccination trials and for covering global needs. In addition, readily neutralized strains suitable for epidemiological work on cat sera, and possibly also for measuring humoral response to vaccines, could be recognized. The extent of serum titer variance, after 10-fold variance of virus input, was established as being only two- to threefold. Five Swiss FCV strains have been shown to remain antigenically stable over 10 years of laboratory passage. FCV strains and the procedure using 20 antibody units offered a suitable approach for international comparative work. FCV strains with little cross-neutralization should be subjected to higher antibody concentrations of existing antisera before attempting to create serotypes. Liver powder absorptions, which reduced homologous and heterologous neutralization titers to similar extents, subsequently proved to be unsuitable for use in FCV classification.

Small feline ether-resistant viruses were originally included as feline picornaviruses in the family *Picornaviridae* (4). Owing to particular biochemical features (3, 5) and a specific ultramorphology (26), they were later classified as caliciviruses. Recently, the Study Group on Picornaviruses deemed it inadvisable to include them in the family *Picornaviridae* (19). Feline caliciviruses (FCV) occur frequently in cats, are isolated with ease in feline cell cultures, and account for a variety of clinical manifestations (4). Their wide range of antigenic overlapping has for many years impeded serological classification and moved them out of the scope of immunoprophylaxis by vaccination.

In 1970 a few workers, convening at the World Health Organization Program on Comparative Virology (25), decided to subject selected and rather well-described FCV strains to a procedure used formerly with success for classification of human enteroviruses (7) and human rhinoviruses (15). This procedure uses sera at dilutions that contain 20 antibody units (AU) and was applied first by our group to five old laboratory strains of FCV of Swiss origin. Based on the results obtained, we then subjected 13 recent Austrian isolates of FCV to selected antisera at 20-AU strength.

In addition, liver powder absorption of Swiss and American antisera against FCV was performed, applying a procedure that had been successfully used to raise the specificity of sera for classification of human rhinoviruses (8).

In this paper we describe the results obtained in these three investigations and discuss their practical implications for future vaccine production, as well as for further international comparative work on calicivirus classification.

MATERIALS AND METHODS

Viruses. Five previously described FCV strains of Swiss origin, 337/61, 344/61, 377/61, 135/62, and 227/62 (3), were used. They had been plaque-purified and were in in vitro passages 13 to 14, with titers ranging from $10^{6.0}$ to $10^{7.8}$/0.1 ml.

In addition, the 13 recent Austrian FCV isolates listed in Table 2 were used. All had been isolated by our laboratory, 11 from pharyngeal swabs of sick or latently infected cats on primary feline kidney cells and 2 that were pick-ups recovered from such cell cultures harboring latent viruses. All 13 isolates were tested without purification in tissue culture passages 1 to 3.

Antisera. Antisera against the five Swiss FCV strains were prepared in rabbits by giving them two inoculations of virus harvests spaced 6 weeks apart, followed by bleeding 1 week later.

Antisera against the five American FCV strains, F 10, F 11, F 17, 5 FPL, and CFI (kindly supplied by J. Parker, Microbiological Associates, Bethesda, Md.), and the Australian pick-up strain KCD had been prepared in goats by J. L. Bittle, Pitman-Moore Inc., Fort Washington, Pa. (National Cancer Institute Contract). The history of these strains and the production of their respective antisera have been published by other authors (14).

Liver powder absorption of sera. Lyophilized liver powder (kindly prepared by J. Kerschbaum, Sanabo G.m.b.H., Vienna) was produced from livers of healthy young cats according to Conant and Hamparian (8). After seven consecutive washings with phosphate-buffered saline, pH 7.4 (8), three sequential absorptions were made, followed by ultracentrifugation and ultrafiltration through membrane filter pads of 0.22-μm pore diameter (Millipore Corp.). This latter step proved necessary to clear the absorbed sera from opalescent debris not removed by the recommended ultracentrifugation (30 min at 30,000 $\times$ g). Without ultrafiltration, absorbed sera were considerably toxic; thereafter, however, even serum diluted 1:4 was well tolerated by cell cultures.

Neutralization tests. Starting with a basic dilution of 1:8, sera were diluted in twofold steps with phosphate-buffered saline, pH 7.4, mixed with 100 50% tissue culture infective doses ($TCID_{50}$) of virus (determined according to Kärber [16]) per 0.1 ml, and incubated at 37°C in a water bath for 60 min. Thereafter, 0.2 ml of each mixture was dispensed into four tube cultures. Virus controls, serum controls, and cell control were included in each test. Readings were made daily until the recorded amount of $TCID_{50}$ was established and occasionally for 2 additional days to assure that no additional virus was detected. One AU was contained in the highest serum dilution neutralizing 32 to 320 $TCID_{50}$ of the homologous virus strain; 20 AU was determined to be the 20-fold-lower dilution of the same serum)15).

Cell cultures. The feline CRFK cell line of Crandell et al. (10), certified as free from *Mycoplasma* (kindly supplied by C. G. Fabricant, Cornell University, Ithaca, N.Y.), was used throughout this work. Fetal instead of normal calf serum was used in the growth medium, with 2% in the maintenance medium and Earle balanced salt solution instead of the described (10) Hanks balanced salt solution.

RESULTS

Cross-neutralization tests with 20 AU. As evident from Table 1, the use of 20 AU effected homologous and far-reaching heterologous neutralization among five Swiss FCV strains and their respective antisera from rabbits. Heterologous neutralization was designated as complete if cytopathic effect was suppressed completely on the day of the last reading and as partial if less than four inoculated tubes remained free of cytopathic effect at that time.

TABLE 1. *Cross-neutralization results between five Swiss FCV strains and their respective antisera from rabbits used at a 20-AU dilution*

Virus		Cross-neutralization[a] with antiserum:				
Strain	$TCID_{50}$	337/61 (20 AU)	344/61 (20 AU)	377/61 (4 AU[b])	135/62 (20 AU)	227/62 (20 AU)
337/61	180	+	p	+	−	+
344/61	1,800	+	+	+	−	+
377/61	320	−	−	+	−	+
135/62	320	−	−	p	+	+
227/62	320	−	−	+	−	+

[a] +, Complete neutralization; p, partial neutralization; −, no neutralization.

[b] Because of low potency, only 4 AU could be used with this serum.

Antiserum 227/62 was able to cross-neutralize all strains included in the test. Antiserum 377/61 almost duplicated it, which is remarkable, because due to its low potency it could only be used at 4-AU strength. Based on these results and relying on other workers' interpretations (7, 11, 15), we consider these two strains to be identical.

Antisera 337/61 and 344/61 showed complete and partial cross-neutralization, respectively. In addition, both strains were fully neutralized by antisera 227/61 and 377/61. Strains 337/61 and 344/61 must be closely related antigenically, but are not identical according to definition (14) and to the results in Table 3.

Antiserum 135/62 failed to cross-neutralize, although reciprocally strain 135/62 was completely neutralized by antiserum 227/62.

Based on these results and drawing parallels to interpretations by authors classifying picornaviruses, our FCV strain 227/62 is a prime strain with a broad antigenic spectrum whereas 135/62 is a strain diffficult to neutralize (7, 24). A comment on the other strains is given below.

Attempts to classify 13 recent FCV isolates through cross-neutralization, as documented in Table 2, clearly showed that antiserum 227/62 was the crucial serum for such purposes. Without exception, 32 to 320 $TCID_{50}$ of these 13 isolates were completely cross-neutralized by 20 AU of antiserum 227/62.

Antisera 344/61 and 135/62, each 20 AU strong, were included in this test series to investigate further their antigenic position among European FCV strains. The purely one-sided cross-neutralization observed in Table 1 for antiserum 135/62 may have resulted from its antigenic composition and thus reflects an ac-

tual finding, or it may have resulted from the small number of strains tested initially and thus is a product of chance.

Table 2 substantiates that the former assumption holds true. Failure of 20 AU to cross-neutralize 10 Austrian FCV isolates and to cross-neutralize three other strains only partially documents that strain 135/62, and its antiserum, truly has a narrow spectrum.

On the other hand, antiserum 344/61 gave a partial neutralization of 6 and a complete neutralization of 1 among 13 Austrian isolates, reflecting the intermediate antigenic broadness of this strain (see Table 1).

Cross-neutralizing with liver powder-absorbed sera. Four of the five Swiss strains and their respective antisera were subjected to studies with liver powder-absorbed sera. Strain 377/61 was omitted because of probable identity with 227/62 and the low potency of its antiserum.

Recorded in Table 3 are the results obtained with unabsorbed sera and with the same sera after a threefold absorption with feline liver powder. When unabsorbed, antiserum 227/62 performed as in Table 1; i.e., it cross-neutralized to high titer all strains involved. The three other antisera, however, displayed remarkably more cross-neutralizations in Table 3 than in Table 1. The most striking observation was the cross-reactivity of 135/62 and its antiserum against all other strains and sera brought forth by higher antibody concentrations. Antiserum 135/62 had been used at a dilution of 1:204.5, corresponding to 20 AU, for the results given in Table 1. The lowest dilution, 1:8, used for the tests in Table 3, however, corresponds with 512 AU. Some additional and contradictory cross-neutralizations recorded in Table 3, as against those in Table 1, result from the use of different virus inputs (see below).

Three absorptions with feline liver powder dramatically reduced previous neutralization titers in all sera (Table 3). The homologous titers were depressed 64-, 32-, and twice 16-fold, respectively. The heterologous titers were reduced 16-fold (3×), 8-fold (1×), or beyond possible quantitation.

Titers persisting after three absorptions were limited with three sera to the homologous strain. On the contrary, with antiserum 227/62, directed against the broadest antigenic strain, significantly higher neutralization persisted against the three heterologous than against the homologous strain.

Cross-neutralization titers of six American antisera against four Swiss strains are recorded in Table 4. As long as they are unabsorbed, all six American sera cross-neutralized at medium to high titer levels with two or three of the four FCV strains. Only Swiss strain 135/62 was exempted from cross-neutralization, sustaining its poor susceptibility already shown in Table 1.

Apart from antiserum 5 FPL, American antisera (in Table 4) showed titers of the same magnitude as those of the European antisera in Table 3.

After three absorptions with feline liver powder, cross-neutralization titers of American sera dropped 64- (3×), 32- (4×), 16- (3×), 8-

TABLE 2. *Attempt to classify 13 recent Austrian FCV isolates using 20 AU of selected antisera from rabbits*

Virus isolates		Neutralization[a] with antiserum:		
Strain	$TCID_{50}$	227/62	344/61	135/62
18/67	100	+	–	–
27/67	100	+	p	p
35/67	100	+	p	p
195/67	320	+	–	–
196/67	100	+	–	–
200/67	56	+	p	–
202/67	32	+	p	p
234/67	100	+	+	–
253/67	56	+	–	–
256/67	56	+	p	–
259/67	100	+	p	–
152/71	180	+	–	–
1382/71	100	+	–	–

[a] +, Complete neutralization; p, partial neutralization; –, no neutralization.

TABLE 3. *Homologous and cross-neutralization titers against four European FCV strains with rabbit sera in the unabsorbed and the liver powder-absorbed states*

Virus		Titer with serum:							
		337/61		344/61		135/62		227/62	
Strain	$TCID_{50}$	Unabsorbed	Absorbed	Unabsorbed	Absorbed	Unabsorbed	Absorbed	Unabsorbed	Absorbed
337/61	100–180	1,024	16	<8	<8	8	<8	1,024	64
344/61	100–180	1,024	<8	256	16	128	<8	512	32
135/62	56–320	32	<8	32	<8	4,096	256	256	32
227/62	100–320	128	<8	8	<8	64	<8	512	8

TABLE 4. *Cross-neutralization titers between four European FCV strains and six American antisera from goats in the unabsorbed and the liver powder-absorbed states*

Virus		Titer with serum:											
		F 17		F 10		KCD		CFI		F 11		5 FPL	
Strain	$TCID_{50}$	Unabsorbed	Absorbed	Unabsorbed	Absorbed	Unabsorbed	Absorbed	Unabsorbed	Absorbed	Unabsorbed	Absorbed	Unabsorbed	Absorbed
337/61	32-320	4,096	64	256	8	512	8	2,048	128	256	16	8	NT[a]
344/61	180	256	16	512	16	256	16	32	8	256	8	64	8
135/62	180	<8	NT	<8	NT	<8	NT	<8	NT	<8	NT	<8	NT
227/62	100-320	1,024	16	256	8	<8	NT	<8	NT	<8	NT	64	<8

[a] NT, Not tested.

(1×), and 4-fold (1×), respectively, in sera with residual activity. In others, showing no cross-neutralization at a 1:8 dilution, the extent of titer reduction could not be determined. Titers persisting after absorptions did not reveal any specificity between a given strain and antisera tested, or vice-versa.

Effect of virus concentration on heterologous titers of unabsorbed sera. As evident from Tables 3 and 4, a number of cross-neutralizations could not be done within one single test for a given virus strain. All results recorded are well within the prescribed input of 32 to 320 $TCID_{50}$ (15). Nevertheless, it seemed desirable to investigate further to what extent cross-neutralization was affected between a given virus and a heterologous antiserum by using different virus inputs. As could have been expected, invariably lower heterologous titers were established with rising numbers of $TCID_{50}$. It is remarkable that a straight-line linear regression with a steep slope results when virus and antiserum are plotted on a logarithmic scale (Fig. 1). Such regressions had been described earlier for homologous neutralization of poliovirus (17) and of bacteriophage T_3 (23). Further on, the steepness of both slopes reflects the small differences observed in Table 5 between serum titers obtained with the fairly large virus input differences used (23).

When evaluating the results of Tables 3 and 4 using the same parameters, titers of unabsorbed sera recorded may have been influenced by a factor of 2 to 3 by a difference in virus input. Its influence on the results obtained and their interpretation are discussed below.

DISCUSSION

Several authors (see 14) attempted in previous years to classify FCV strains by subjecting their cross-neutralization results to the formula of Archetti and Horsfall (1). A World Health Organization Board on Comparative Virology (25), however, judged this approach to be unsuitable for classification of FCV strains, because even minor antigenic differences, so frequent among these strains, sufficed to simulate new serotypes. Another approach (6), namely, determination of residual virus titers persisting after cross-neutralization, followed by their biomathematical analysis, did not furnish defined serotypes either.

Attempts to classify FCV strains by complement fixation (13, 21, 22), precipitation methods (14, 22), or immunofluorescence (12) all were unsuccessful in that they revealed much wider cross-reactions than did cross-neutralization tests.

Evidently, then, working with 20 AU in cross-neutralization tests brought an improvement in previous serological classification of FCV strains. When demonstrated, cross-neutralization with 20 AU was one- or two-sided. Its extent was far smaller than that shown in Table 3, where the same antisera were used in high concentrations.

Each procedure used, however, has its merit. When working with 20 AU, strain 227/62 could be determined as a prime strain with broad antigenicity. Its antiserum not only neutralized all 5 Swiss FCV strains (Table 1), but invariably 13 recent Austrian FCV isolates also (Table 2).

Of course, concentrations higher than 20 AU, as used for the results shown in Table 3, confirmed the cross-reactions recorded in Table 1. In addition, further cross-reactivity was disclosed. Neither procedure permitted the segregation of serotypes (see below), but permitted conclusions of practical implication.

Strain 227/62 is clearly the candidate of choice as a future vaccine strain for European use (Tables 1 and 2). It could be determined more clearly with the 20-AU test procedure.

Recognition of the easily cross-neutralized strain 344/61, suitable for epidemiological work, was possible with the 20-AU method (Table 1) as well as with the conventional serum dilution method (Tables 3 and 4).

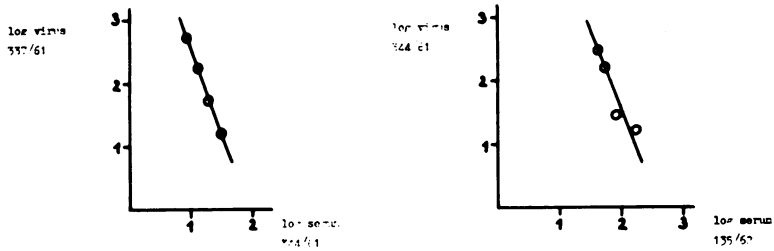

FIG. 1. *Relationship between variable amounts of virus and neutralization titers of two heterologous FCV antisera.*

TABLE 5. *Influence of rising virus input on selected heterologous antisera titers*

Virus		Titer with serum:		
Strain	$TCID_{50}$	337/61	344/61	135/62
337/61	18	NT[a]	32	NT
	56	NT	19	NT
	180	NT	13	NT
	560	NT	9.5.5	NT
344/61	18	NT	NT	181
	32	NT	NT	90
	180	NT	NT	53
	320	NT	NT	45
227/62	18	19	NT	NT
	56	<8	NT	NT
	180	8	NT	NT
	560	<8	NT	NT

[a] NT, Not tested.

Readily neutralized FCV strains, such as strains 344/61, FS (14), and F 19 (20), may be recommended for serological surveys among cats and possibly also for assessment of humoral immune response to vaccination.

When using the 20-AU method only strain 135/62 had to be regarded as a serological variant (25). Evaluating only data from Table 1 would have left undetermined whether its antigenic composition necessitates incorporation into envisaged vaccines. Taking into account the difficulty of neutralizing certain human Echo viruses, it was recommended (24) that 100 to 1,000 AU of existing antisera be used before creating additional Echovirus serotypes. Applied to our FCV strains and antisera, this disclosed that strain 135/62 is a poorly neutralized FCV but has demonstrable two-sided cross-reactivity. Its incorporation into vaccines is, therefore, unnecessary. Cross-reactivity, according to the data in Table 4 and the results of Povey (20), exists also between European and American FCV strains to a remarkable degree.

No strain of our Table 1 fulfills the criteria of a separate serotype, which, according to the rhinovirus committee (15), was defined as follows: "A candidate rhinovirus was considered to be distinct if at least 20 times the limiting concentration of specific antisera which neutralized 32–320 TCD_{50} of the other serotypes (that is 20 A.U.) failed to neutralize 32–320 TCD_{50} of the candidate virus and if at least 20 A.U. of serum to the candidate virus failed to neutralize 32–320 TCD_{50} of each of the other serotypes."

Similar to our results, in two other investigations published recently no separate FCV serotype could be established (14, 19). These authors found the F 9 (14) and FS (20) strains, respectively, to be antigenically broad strains corresponding to our 227/62 strain.

Direct comparison among results now available from three laboratories is impossible, because we, as well as Povey (20), worked with identical 20-AU methods but with different strains, whereas Povey (20) and Kalunda et al. (14) studied identical strains but used different methods.

It becomes, however, apparent that our strain 135/62 is least cross-reactive with Swiss, Austrian, and American test material. Likewise, strain 5 FPL was least cross-reactive in American (14) and in Austrian tests (Table 4).

Accordingly, FCVs make good test objectives for further international comparison, which may be needed because of the detection of caliciviruses in swine, pinnipeds (18), and presumably humans (G. R. Maddeley, personal communication).

Our five Swiss strains have remarkably maintained, over 10 years of laboratory passage, their original (3) cross-neutralization pattern. The steep slopes of the virus titer/serum titer relationship evidenced in Fig. 1 permit the conclusion that variance of virus titers has little impact on serum titers (23). In consequence, FCVs fulfill a prerequisite for future comparative work between cooperating laboratories.

Moreover, the linear regressions demonstrated in Fig. 1 for two heterologous systems

can be considered a further indicator of antigenic identity among the FCV group of viruses in being of the same appearance as those for homologous systems tested by other authors (17, 23).

Absorptions with human liver powder have greatly promoted classification of human rhinoviruses (8, 9, 15). By removal or reduction of nonspecific inhibitors, minor cross-reactions were eliminated in almost all instances. At the same time, homologous neutralization titers remained unaffected or were reduced only fourfold. Liver powder adsorptions permitted reduction to 55 serotypes of human rhinoviruses from 68 candidate strains, with a single subtype remaining for serotype 1. The species of the serum donor was unimportant.

No such beneficial effect was evident after absorptions with feline liver powder of four European antisera produced in rabbits and six American antisera produced in goats. Obviously, in sera of both species a more or less linear regression of both homologous and heterologous neutralization titers was obtained. Their remaining neutralizing activity did not contribute to classification of the four FCV strains tested. Accordingly, no effort was made to determine what kind of substance(s) had been removed by liver powder absorptions.

In conclusion, should any further comparative work on FCV, and possibly caliciviruses from other species, be undertaken, the basic approach should follow the recommendations of the rhinovirus and enterovirus committees (11, 15). Strains remaining unclassified with 20 AU should then be tested with higher antibody concentrations before creating new serotypes.

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