A Pan-Picornavirus RT-PCR: Identification of Novel Picornavirus Species

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A pan-picornavirus one-step RT-PCR was developed based on multiple degenerate, inosine containing, virus-sense primers targeted at the 3Dpol region containing the conserved XXYGDD motif and a single oligo-dT-containing antisense primer. A set of nine forward primers were designed to cover all the possible known picornavirus sequences, however, most picornaviruses were detected by one or more of a subset of three primers. The amplicons produced were between 500 and 600 bp, depending on the length of the 3′ UTR. Although not all currently identified picornaviruses have yet been tested, all those in our collection were amplified by this assay. Additionally, amplicons were produced for a number of candidate novel picornaviruses. Sequencing of the amplicons was achieved initially using the forward primer. Specific primers, adjacent to the poly(A) tail, were then designed for sequencing the amplicons in the reverse direction. Phylogenetic analysis then allowed tentative identification of each of the viruses at the genus and species levels. Comparisons of the sequences of picornavirus 3′ UTR's show them to be diagnostic of species, but not of genus in the case of a new virus species. However, in most cases, sequences at the 3′ end of the 3D^{pol} could be used for genus assignment. The tentative classification of a picornavirus isolated from common (harbour) seals (*Phoca vitulina*) in the North Sea in 1988 in a novel picornavirus genus will be presented. Four acid-stable equine picornavirus isolates, which are seologically distinct from equine rhinitis A virus (ERAV), equine rhinitis B virus 1 (ERBV-1) and ERBV-2, were provisionally identifed as a members of the genus *Erbovirus*. A virus isolated from the heart of a pig with acute myocarditis in Italy was identified as a member of the species *Porcine enterovirus A* (genus *Enterovirus*). A number of other suspected picornaviruses were also identified including the initial identification of three bovine rhinovirus serotypes as probable members of the genus *Aphthovirus* (N.J. Knowles, presented at this meeting). This assay was also used to identify ERAV from cases of abortion in camels (U. Wernery *et al.*, presented at this meeting). This RT-PCR should prove to be of great value in the initial identification of candidate picornaviruses.

Primer Design

Reverse primer for RT-PCR

3′ RACE-A: 3′ - TTTTTTTTTTTTTTTTTTTTCCCGATAAGAGCTCGCTAGCC – 5′

Forward primers for RT-PCR

L	C	Y	G	D	D		
YTI	TGY	TAY	GGI	GAY	GA	(16)	HAV; PEV-A; PTV
I	C	Y	G	D	D		
ATI	TGY	TAY	GGI	GAY	GA	(8)	ERAV
L	T	Y	G	D	D		
YTI	ACI	TAY	GGI	GAY	GA	(8)	AEV; ERBV
L	S	Y	G	D	D		
YTI	WSI	TAY	GGI	GAY	GA	(32)	EMCV; ThV
I	S	Y	G	D	D		
ATI	WSI	TAY	GGI	GAY	GA	(16)	FMDV; SV26/2V35
I	A	Y	G	D	D		
ATI	GCI	TAY	GGI	GAY	GA	(4)	HRV-A; HEV-B; HEV-C; HEV-D; PV; BEV; PEV-B
V	A	Y	G	D	D		
GTI	GCI	TAY	GGI	GAY	GA	(4)	HEV-A; SV43
L	A	Y	G	D	D		
YTI	GCI	TAY	GGI	GAY	GA	(8)	HRV-B; HRV-A; AiV; BKV; DPV; SV2; SV18
I	V	Y	G	D	D		
ATI	GTI	TAY	GGI	GAY	GA	(4)	HPeV; LV

Individual primers were synthesized as above, since a single degenerate primer would have to cope with the following aa sequence: (LIV) (CTSAV) YGDD

Genus / species	Serotype	Strain	ISYGDD	IAYGDD	ICYGDD
Enterovirus					
Poliovirus		not available	nd	nd	nd
Human enterovirus A	CV-A16	GRE/1/75	-	++	-
	EV-71	BrCr	-	++	-
Human enterovirus B	CV-B3	Nancy	-	++	-
	CV-B5	SVDV/UKG/27/72	-	++	++
Human enterovirus C	CV-A24	EH 24/70 (AHC)	-	++	++
Human enterovirus D	EV-70	J670/71 (AHC)	nd	nd	nd
Simian enterovirus A	SEV-A1	SV28	nd	nd	nd
Porcine enterovirus A	PEV-8	V13	nd	nd	nd
Porcine enterovirus B	PEV-9	UKG/410/73	nd	nd	nd
Bovine enterovirus	BEV-1	VG/5/27	-	+	-
	BEV-2	BEV-261	++	++	++
unnamed	SV2	2383	nd	nd	nd
	SV49	2600	-	+	-
Rhinovirus					
Human rhinovirus A		not available	nd	nd	nd
Human rhinovirus B		not available	nd	nd	nd
Cardiovirus					
Encephalomyocarditis virus	EMCV	VR-129B	++	++	-
Theilovirus	TMEV	GDVII	-	+	+
Aphthovirus					
Foot-and-mouth disease virus	FMDV-O	IND/1/62	++	++	++
Equine rhinitis A virus	ERAV	PERV	-	-	+
Hepatovirus					
Hepatitis A virus	HAV	not available	nd	nd	nd
Avian encephalomyelitis virus	AEV	not available	nd	nd	nd
Parechovirus					
Human parechovirus	HPeV-1	Harris	nd	nd	nd
Ljungan virus	LV	87-012	nd	nd	nd
Erbovirus					
Equine rhinitis B virus	ERBV-1	P1436/71	-	+	-
Kobuvirus					
Aichi virus	AiV	A846/88	-	+	-
Bovine kobuvirus	BKV	U-1	nd	nd	nd
Teschovirus					
Porcine teschovirus	PEV-1	Talfan	++	++	++

Unclassified viruses	Strain	ISYGDD	IAYGDD	ICYGDD
bovine rhinovirus 1 (BRV-1)	RS 3x	-	+	+
bovine rhinovirus 2 (BRV-2)	EC 11	-	+	-
bovine rhinovirus 3 (BRV-3)	H-1	+	++	++
sheep enterovirus	VS65.60	-	+	-
sheep enterovirus	VS65.66	nd	+	nd
sheep enterovirus	VS65.80	nd	++	nd
sheep enterovirus	VS65.114	nd	++	nd
sheep enterovirus	990	-	++	-
mystery virus	SVV	-	+	+
Italian pig virus	ITL/1954/99	-	-	++
acid-stable equine picornavirus (EqPV)	4442/75	+	++	-
EqPV	5353	-	+	-
EqPV	R4/4	-	+	-
EqPV	2484C	+	++	-
Unknown camel abortion virus	D1305-03	-	+	++
seal picorna-like virus (SPLV)	Holland/88	-	++	-
duck hepatitis virus 1 (DHV-1)		-	-	-
duck hepatitis virus 2 (DHV-2; astrovirus)		-	-	-

Also see these posters elsewhere at this meeting:

Knowles, N.J. - Molecular identification of all three bovine rhinovirus serotypes as members of the genus *Aphthovirus*.

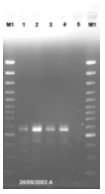
Knowles, N.J. and Hallenbeck, P.L. - A new picornavirus is most closely related to cardioviruses.

Wernery, U., Knowles, N.J., Hamblin, C., Wernery, R., Joseph, S., Kinne, J., Nagy, P. and Graham, S.D. - Abortions in dromedaries caused by equine rhinitis A virus.

Acknowledgement

I would like to thank Roland Zell for the sequence of the 3′-RACE-A primer.

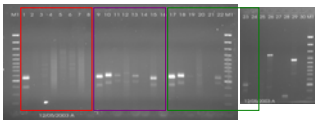
Examples of some of the RT-PCR amplicons obtained



1	Seal picornavirus Vero 7	IAYGDD
2	Seal picornavirus Vero 8	IAYGDD
3	Seal picornavirus Vero 7	IAYGDD (x2)
4	Seal picornavirus Vero 8	IAYGDD (x2)
5	Negative control (water)	IAYGDD

1	++	9	++	17	++	FMDV OS/IND/1/62
2	-	10	++	18	++	SVDV UKG/27/72
3	-	11	+	19	+ f	TMEV GDVII
4	-	12	+ f	20	-	ERBV-1 P1436/71
5	-	13	+	21	-	ShEV VS65.60
6	-	14	-	22	+	ITL/1954/99
7	-	15	+	23	+	SVV
8	-	16	-	24	-	Neg control

ISYGDD/3′-RACE-A IAYGDD/3′-RACE-A ICYGDD/3′-RACE-A



METHOD

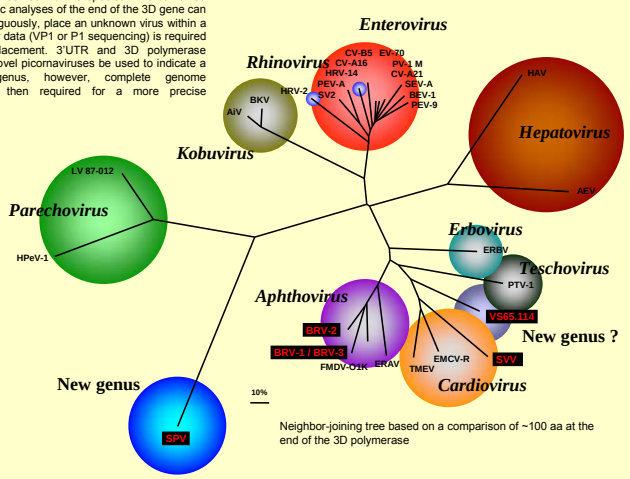
Total RNA was extracted from cell culture supernatants using QiaGen's RNeasy spin-columns.

Amplification was carried out using Amersham's Ready-To-Go One-Step RT-PCR beads
- 40 pmoles reverse primer
- 20 pmoles forward primer
- 5-10 µl RNA
- Thermal profile:
  - 42 °C for 30 min
  - 94 °C for 5 min
  - 35 to 45 cycles of 94 °C for 1 min, 45 °C for 1 min, 72 °C for 1.5 min
  - 72 °C for 5 min

Sequencing was carried out on the Beckman CEQ8000 using the forward primer and later specific reverse primers at the 3′ end of the genome.

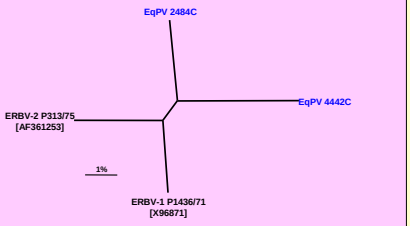
Identification of New Picornavirus Species

Picornaviruses have unique 3′ UTR sequences which are normally well conserved within a species. This combined with phylogenetic analyses of the end of the 3D gene can usually, unambiguously, place an unknown virus within a species. Further data (VP1 or P1 sequencing) is required for serotype placement. 3'UTR and 3D polymerase sequences of novel picornaviruses be used to indicate a new species/genus, however, complete genome sequencing is then required for a more precise classification.



Neighbor-joining tree based on a comparison of ~100 aa at the end of the 3D polymerase

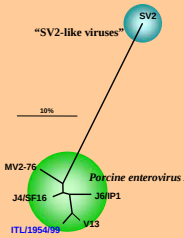
Acid-stable equine picornaviruses (EqPV)



During 1974/75 twelve acid stable picornaviruses were isolated from nasopharyngeal swabs taken from horses in England (Mumford and Thomson, 1978). Ten of the viruses were isolated from a single, healthy animal over a period of 18 months. Four of the isolates which were compared in VN tests belonged to a single serotype, but were not identical. The virus isolate 4442/75 was 22 nm in diameter, possessed a RNA genome, was resistant to chloroform, rapidly inactivated at 50 °C, but stabilized by 1M $MgCl_2$. It was stable at pH 3.1 – 7.0 but rapidly inactivated at pH 2.7. The buoyant density in CsCl was 1.40 - 1.43 g/ml. EqPV/4442/75 grew, producing CPE, on RK-13, IB-RS-2 and LLC-MK2 cells. Viruses kindly provided by Jenny Mumford, Animal Health Trust, Newmarket.

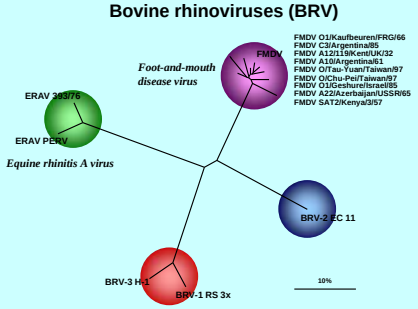
The acid-stable EqPVs appear to be members of the genus *Erbovirus*, species *Equine rhinitis B virus*, and probably represent a third serotype. Neighbor-joining tree based on a comparison of 544 nt at the 3′ end of the genome.

Italian pig virus



ITL/1954/99 isolated from pigs with myocardial lesions in Italy during investigations of EMCV-related disease. Shows CPE on BHK-21 & IB-RS-2 cells. Enterovirus-like particles were observed by EM. The virus was provided by Emiliana Brocchi, Brescia, Italy.

Bovine rhinoviruses (BRV)



See poster on Bovine rhinoviruses

Seal picornavirus (SPV)

Relationship of the seal picornavirus to other members of the *Picornaviridae*

HEV-D	25.0	PEV-A	20.0
LV	24.5	PTV-1	19.4
PV	24.0	EMCV	19.2
HEV-C	24.0	ERBV	18.6
SV2	22.9	HRV-A	18.3
HEV-B	22.1	FMDV	17.6
HEV-A	22.1	ERAV	16.5
SEV-A	22.1	TMEV	16.0
HPeV	21.7	AiV	15.7
BEV	21.2	BKV	15.6
PEV-B	21.2	HAV	14.4
HRV-B	21.2	AEV	13.9

% amino acid identities at the end of the 3D polymerase (~130 aa)

Isolated from a harbour or common seal off the coast of Holland in 1988 during the seal plague epidemic. Grows on Vero, LLC-MK2 and BHK-21 cells. Virus provided by Jan Groen, Netherlands.