Genetic Relationships Between Bovine Enteroviruses in VP1 and 3D^pol^

Nick J. Knowles

Institute for Animal Health, Pirbright Laboratory, Pirbright, Woking, Surrey, UK. E-mail: nick.knowles@bbsrc.ac.uk

BACKGROUND

The bovine enteroviruses (BEV) classification scheme of Dunne *et al.* (1971) designated eight serotypes, which included four previously described by Barya *et al.* (1967) (Table 3). However, it was later discovered that Dunne's type 5 and type 8 were identical (S.B. Mohanty, personal communication, 1980). Subsequently, Knowles and Barnett (1985), using Dunne's seven prototype strains, showed type 3 and type 7 to be identical and types 1, 4, 5 and 6 to be members of a single serotype, by VN, CF and IP tests. Knowles and Barnett (1985) included in their two-serotype classification seven BEV serotypes described by Huck and Cartwright (1964) (Table 6). It has long been recognised that within each serotype substantial antigenic variation occurs and it has even been suggested that some strains of type 2 may represent a third serotype (Knowles and Barnett, 1985). Many of the early BEV classification schemes consisted of eight to ten serotypes, while others grouped viruses together into two types. The methods used to produce the antisera for these studies may have been a critical factor in the type of number of serotypes in the various classifications. Until now there has been little sequence analysis of BEV's (Earle *et al.*, 1988; McIlhatton *et al.*, 1993; McNally *et al.*, 1994; Goens *et al.*, 2004) and the later two of these have provided substantially different sequences for the same isolate, PSU87. Therefore, to gain an insight into the genetic variation of these viruses, various RT-PCR's were developed and the resultant amplicons sequenced.

MATERIALS AND METHODS

One-step RT-PCR's RT-PCR's were developed to amplify three regions of the BEV genome: i) the 5' untranslated region (UTR); ii) the VP1 capsid-coding region; and iii) the 3' end of the genome (Tables 1 and 2). To examine the VP1 gene, one-step RT-PCR's were performed using two different primer sets (Table 2). 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 and 5-10 µl RNA; thermal cycling profile: 42 °C for 30 min, 94 °C for 5 min, 35 cycles of 94 °C for 1 min, 50-55 °C (see Table 2) for 1 min, 72 °C for 1.5 min and 72 °C for 5 min. Automated cycle sequencing was carried out on a Beckman CEQ8000 using their recommended reagents and protocols. Unrooted Neighbor-joining trees were constructed using PHYLIP 3.5c (Felsenstein, 1993) and rendered using TreeView 1.6.6 (Page, 1996). Various other BEV strains (McFerran, 1958, 1962) were also amplified in the three assays, but only the 3D region amplicons have been so far sequenced. Some porcine enteroviruses were included in the analyses.

RESULTS and DISCUSSION

All the BEV's studied were amplified by the 5' UTR primer set (data not shown), however, these amplicons have not yet been sequenced. Sequence analysis of both the VP1 and 3D^pol^ genome regions revealed two distinct clusters correlating with the two serotypes (Figs. 2 and 3). However, analysis of the predicted VP1 amino acid sequences revealed additional clustering within each serotype which was not observed in the nucleotide trees (Fig. 4). BEV type 1 contained two main clusters, i) strains VG/5/27, BEV-1, M63 and ii) strains BEV-165, PSU83, PSU42, T10, M153, K2577, SL305 and M6. BEV type 2 strains were even more varied forming five clusters: i) BEV-261 and UKG/98/79(a); ii) PSU89, PSU87 (Pirbright & Maryland) and 3A; iii) F266a, PSU87 (Belfast) and M134; iv) M80; and v) T11f. These genetic clusters are similar to the antigenic subtypes described by Knowles and Barnett (1985) and may represent natural antigenic groups which could form the basis of a new serotype classification. When comparing the two genome region studied, no evidence of recombination between BEV-1 and BEV-2 was observed. Thus it may be possible to designate BEV-1 and BEV-2 as two distinct species with the enterovirus genus.

	RNA	Forward primer	Reverse primer
1	BEV-1 LCR4 (VR-248)	BEV-1C587F	BEV-2A82R
2	BEV-1 M4 (VR-756)	BEV-1C587F	BEV-2A82R
3	BEV-1 PS 83 (VR-757)	BEV-1C587F	BEV-2A82R
4	BEV-1 PS 83 (VR-757)	BEV-1C587F	BEV-2A82R
5	BEV-1 PS 42 (VR-758)	BEV-1C587F	BEV-2A82R
6	Negative control (water)	BEV-1C587F	BEV-2A82R
7	BEV-2 M2 (VR-754)	BEV-1C587F	BEV2-2A97R
8	BEV-2 PS 89 (VR-755)	BEV-1C587F	BEV2-2A97R
9	BEV-2 PS 87 (VR-774)	BEV-1C587F	BEV2-2A97R
10	Negative control (water)	BEV-1C587F	BEV2-2A97R
11	BEV-1 LCR4 (VR-248)	BEV-3DF	3'-RACE-A
12	BEV-1 M4 (VR-756)	BEV-3DF	3'-RACE-A
13	BEV-1 PS 83 (VR-757)	BEV-3DF	3'-RACE-A
14	BEV-1 PS 83 (VR-757)	BEV-3DF	3'-RACE-A
15	BEV-1 PS 42 (VR-758)	BEV-3DF	3'-RACE-A
16	BEV-2 M2 (VR-754)	BEV-3DF	3'-RACE-A
17	BEV-2 PS 89 (VR-755)	BEV-3DF	3'-RACE-A
18	BEV-2 PS 87 (VR-774)	BEV-3DF	3'-RACE-A
19	Negative control (water)	BEV-3DF	3'-RACE-A

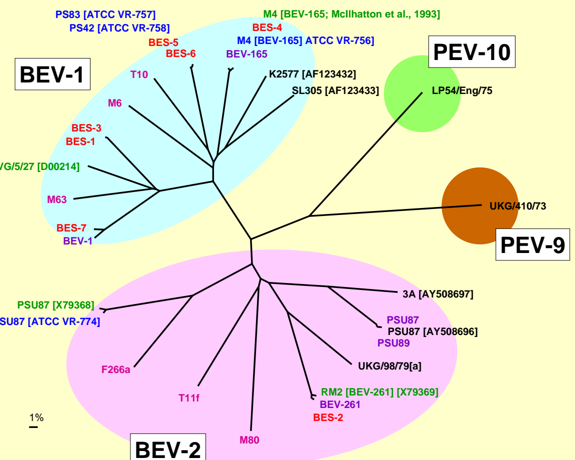


Fig. 2. Neighbor-joining tree showing the relationships between BEV's in the VP1 gene.

Table 1. Oligonucleotide primers used for RT-PCR and sequencing.

Primer name	Primer sequence (5' to 3')	Genome location on VG/5/27 [D00214]
Entero-1F	GTACCTTTGTACGCCTGTT	66-84
Entero-1R	AGGATTAGCCGCATTCA	537-553
BEV-1C587F	CCATGTGGTAYCARACIAAYATGGT	2353-2377
BEV-2A82R	GATTGCCAIACTTCATTYTCCCA	3420-3442
BEV2-2A97R	AGRAGGTCYCTCTCGTAIGAGTCCCA	3435-3460
BEV-1D751R	GTAYACYCCAGTGTAYCKKGACTTGTA	3261-3287
BEV-3DF	TTTGGITTTGAYTAYACIGCITATGATGC	6648-6676
3'-RACE-A	CCGATCGCTCGAGAATAGCCCTTTTTTTTTTTTTTTTTTTT	7415-7457

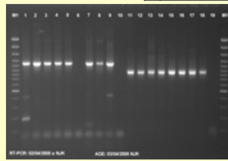


Fig. 1. Example of the results obtained with the various RT-PCR's.

Fig. 3 labels:
VG/5/27 [D00214]
PS83 [ATCC VR-757]
VG/5/31
LCR4 [ATCC VR-248]
BES-1
BES-3
PS42 [ATCC VR-758]
PSU42
PSU83
BES-2
M63
M6
BES-7
MZ468 [D84065]
Glasgow virus
M4 [BEV-165] [ATCC VR-756]
M4 [BEV-165; McIlhatton et al., 1993]
K2577 [AF123432]
03-1658
VJ19/UK/2005
SL305 [AF123433]
ECBO SA
BEV-1
T10
TAN/140/X/68
BOT/209/67
17042
Sh825R [sheep]
IND-13 [dog]
PEV-10
LP54/Eng/75 [AF363455]
CJ87 (UK/87)
PEV-9
Pd72 (Italy/62)
UKG/410/73 [AF363453]
PSU89
PSU87
PSU87 [AY508696]
PS89 [ATCC VR-755]
BEV-2
PS87 [ATCC VR-774]
M2 [BEV-261] [ATCC VR-754]
BEV-261
ECBO 48
3A [AY508697]
UKG/98/79[a]
T11f
03-6888
M134
F266a
M80
VP/7/19
VH7
VH2
VG/5/23
1%

Fig. 3. Neighbor-joining tree showing the relationships between BEV's in the 3D^pol^ gene.

Table 2. Primer pairs used for RT-PCR and amplicons sizes.

Region	Forward primer	Reverse primer	Annealing temp.	Size (bp)
5' UTR	Entero-1F	Entero-1R	50 °C	488
VP1	BEV-1C587F	BEV-2A82R	55 °C	1090
VP1	BEV-1C587F	BEV2-2A97R	55 °C	1108
VP1 (partial)	BEV-1C587F	BEV-1D751R	55 °C	935
3D+3'UTR	BEV-3DF	3'-RACE-A	55 °C	810

Table 3. Viruses received from Professor S.B. Mohanty, University of Maryland, USA, c. 1978

Classification of Dunne *et al.*, 1971	Classification of Knowles *et al.*, 1979	Strain	Sequencing suggests:	
			VP1	3D
BEV-1	BEV-1	BEV-1	BEV-1	Not done
BEV-2	BEV-2	BEV-261	BEV-261	BEV-261
BEV-3	BEV-2	PSU89	PSU89	PSU89
BEV-4	BEV-1	BEV-165	BEV-165	Not done
BEV-5	BEV-1	PSU83	PSU83	PSU83
BEV-6	BEV-1	PSU42	PSU42	PSU42
BEV-7	BEV-2	PSU87	PSU89	PSU89

Table 4. Viruses received from Brian Adair, Stormont, Belfast, September 2003 (ex. Howard Dunne, Pennsylvania State University).

Serotype	Presumed strain name*	Sequencing suggests:	
		VP1	3D
BES 1	BEV-1	LCR4	LCR4
BES 2	BEV-261	BEV-261	PSU83
BES 3	PSU89	LCR4	LCR4
BES 4	BEV-165	BEV-165	not done
BES 5	PSU83	PSU83 or PSU42	PSU83 or PSU42
BES 6	PSU42	PSU83 or PSU42	PSU83 or PSU42
BES 7	PSU87	BEV-1	BEV-1?

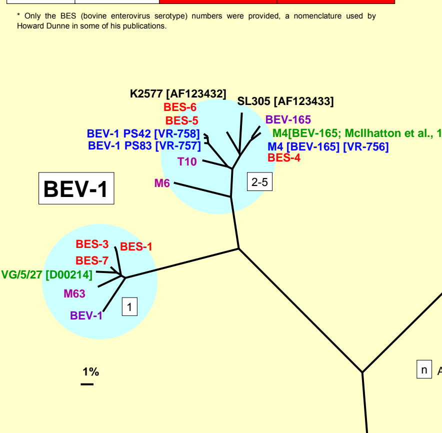
* Only the BES (bovine enterovirus serotype) numbers were provided, a nomenclature used by Howard Dunne in some of his publications.

Cross-neutralization data (Brian McFerran, 1976)

BES 1, 2, 3, 4, 5, 6, and 7. - These are US isolates from H.W. Dunne, Penn State Univ. Cross neutralisation between the BES viruses and N. Ireland strains: BES 1 and 7 are related and show crossing with VG/5/27; BES 2 crosses with VH2; BES3 crosses with VG/5/23; BES4 crosses with BES 1, BES 5, BES 7 and VG/5/27; BES 5 crosses with BES 1, 4, 5, 7, and VG/5/27; BES 6 crosses with BES 1, 4, 5, 6, 7 and VG/5/27; and BES 7 crosses with BES 1 and VG/5/27. – Brian Adair, personal communication, 2003.

Table 5. ATCC BEV prototype strains received from Liz Hoey, Queens University, Belfast, September 2004

BEV type	Strain	ATCC		Passaging at QUB	Sequencing suggests:	
		No.	Passage history		VP1	3D
1	LCR4	VR-248	Steer K/6 MkK/BT (CRL 1309)/1 (Lot #2)	BHK2 19/01/88	Not done	LCR4
2	M2	VR-754	BK/9 (Lot #1)	BHK2 04/06/89	M2 [BEV-261]	M2 [BEV-261]
3	PS89	VR-755	BK/27 (Lot #1)	BHK1 04/12/87	PSU89	PSU89
4	M4	VR-756	BK/9 (Lot #1)	BHK2 BHKpp2 BHK1 07/07/88	M4 [BEV-165]	M4 [BEV-165]
5	PS83	VR-757	BK/23 (Lot #1)	BHK2 BHKpp2 BHK1 11/11/88	PSU83	VG/5/27
6	PS42	VR-758	BK/19 (Lot #1)	BHK2 15/02/88	PSU42	PSU42
7	PS87	VR-774	BK/15 (Lot #1)	BHK2 BHKpp3 BHK2 19/04/90	PSU87	PSU87



n Antigenic subgroup (Knowles & Barnett, 1985)

3
M80
PSU87 [X79368]
PSU87 [ATCC VR-774]
2
T11f
4
F266a
1
BES-2
RM2 [BEV-261] [X79369]
BEV-261
UKG/98/79[a]
5
PSU87
3A [AY508697]
PSU89
PSU87 [AY508696]
BEV-2

Fig. 4. Neighbor-joining tree showing the relationships between BEV's in VP1.

Table 6. Viruses received from Dr. J.W. Harkness, Central Veterinary Laboratory, New Haw, Weybridge, Surrey, England, c. 1970's

Classification of Knowles *et al.*, 1979	Strain	Sequence classification	
		VP1	3D
BEV-1	M6	BEV-1	BEV-1
BEV-1	M63	BEV-1	BEV-1
BEV-2	M80	BEV-2	BEV-2
Not done	M134	BEV-2*	BEV-2
BEV-1	M153	BEV-1*	BEV-1*
BEV-1	T10	BEV-1	BEV-1
BEV-2	T11f	BEV-2	BEV-2
BEV-2	F266a	BEV-2	BEV-2

* Based on partial sequences (data not shown)

CONCLUSIONS

Phylogenetic analysis showed that the bovine enteroviruses examined fell into two genetic clusters apparently consistent with the two recognised serotypes. These two clusters were even more evident when the predicted amino acid sequences were compared. There are clearly some anomalies in the sequencing of the prototype reference strains. This may result from some of the stocks being mixtures of viruses. These may have been present in the original isolates or stocks may have been cross-contaminated in the laboratory at a later time. Further sequencing is required to clarify some of these issues, preferably after plaque purification of the various stocks. Purified and molecularly characterized stocks could then be made generally available and submitted to the ATCC.

ACKNOWLEDGEMENTS

I would particularly like to thank the following for viruses, information and useful discussions: Liz Hoey (Queen's University, Belfast) and Brian Adair and David Graham (Dept. of Agriculture and Rural Development, Veterinary Sciences Division, Stormont, Belfast).

References

- Barya, M.A., Moll, T. and Mattson, D.E. (1967). Antigenic analysis of bovine enteroviruses through studies of the kinetics of neutralization. Am. J. vet. Res. 28: 1283-1294.
- Earle, J.A.P., Skuce, R.A., Fleming, C.S., Hoey, E.M. and Martin, S.J. (1988). The complete nucleotide sequence of a bovine enterovirus. J. gen. Virol. 69: 253-263.
- Dunne, H.W., Wang, J.T. and Ammerman, E.H. (1971). Further studies on the classification of North American porcine enteroviruses: A comparison with European and Japanese strains. Infect. Immun. 4: 619-631.
- Felsenstein, J. (1993). PHYLIP (Phylogeny Inference Package) version 3.5c. Distributed by the author. Department of Genetics, University of Washington, Seattle, 1993.
- Goens, S.D., Botero, S., Zemla, A., Zhou, C.E. and Perdue, M.L. (2004). Bovine enterovirus 2: complete genomic sequence and molecular modelling of a reference strain and a wild-type isolate from endemically infected US cattle. J. gen. Virol. 85: 3195-3203.
- Huck, R.A. and Cartwright, S.F. (1964). Isolation and classification of viruses from cattle during outbreaks of mucosal or respiratory disease and from herds with reproductive disorders. J. comp. Path. 74: 346-365.
- Knowles, N.J. and Barnett, I.T.R. (1985). A serological classification of bovine enteroviruses. Arch. Virol. 83: 141-155.
- McFerran, J.B. (1958). ECBO viruses of cattle. Vet. Rec. 70: 999.
- McFerran, J.B. (1962). Bovine enteroviruses. Ann. N.Y. Acad. Sci. 101: 436-443.
- McIlhatton, M., Hoey, E.M. and Martin, S.J. (1993). The nucleotide sequence of bovine enterovirus strain M4. Abstract of a poster presented at the IXth International Congress of Virology, Glasgow, Scotland, 8-13 August 1993: p. 180, P19-20. Glasgow: ICV.
- McNally, R.M.P., Earle, J.A.P., McIlhatton, M., Hoey, E.M., Martin, S.J. (1994). The nucleotide sequence of the 5' non-coding and capsid coding genome regions of two bovine enterovirus strains. Arch. Virol. 139: 287-299.
- Page, R.D.M. (1996). TREEVIEW: An application to display phylogenetic trees on personal computers. Computer Applications in the Biosciences 12: 357-358.