Three capsid amino acids notably influence coxsackie B3 virus stability

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Coxsackievirus B3 strain 28 (CVB3/28) is less stable at 37 °C than eight other CVB3 strains with which it has been compared, including four in this study. In a variant CVB3/28 population selected for increased stability at 37 °C, the capsid proteins of the stable variant differed from the parental CVB3/28 by two mutations in Vp1 and one mutation in Vp3, each of which resulted in altered protein sequences. Each of the amino acid changes was individually associated with a more stable virus. Competition between CVB3/28 and a more stable derivative of the strain showed that propagation of the less stable virus was favoured in receptor-rich HeLa cells.

INTRODUCTION

Coxsackievirus B3 (CVB3; family Picornaviridae, species Human enterovirus B) strains that differ in their cytopathic effects on HeLa and RD cells have different stabilities at 37 °C as well as varied abilities to bind secondary ligands (Carson et al., 2011). The infectious half-lives when incubated at 37 °C range from 7 h for CVB3/28, propagated in coxsackievirus and adenovirus receptor (CAR)-rich HeLa cells, to 14 h for CVB3/RDVA, propagated in CAR-poor RD cells (Carson et al., 2007, 2011). This observation led to the hypothesis that more stable CVB3 strains are favoured when infection is less efficient due to decreased CAR availability on host cells. CVB3/28, the strain most commonly used in our laboratories (Tracy et al., 2002), replicates very well in CAR-rich HeLa cells and has the shortest half-life that we have yet measured among different CVB3 strains (Carson et al., 2011; Organtini et al., 2014). CVB3/RD, which is more stable than CVB3/28, emerged when CVB3/M was serially propagated in CAR-poor RD cells (Reagan et al., 1984). Furthermore, the strain designated CVB3/RDVa, isolated from a CVB3/RD carrier culture in RD cells, is even more stable (Carson et al., 2011). Based on these observations, we hypothesized that CVB3/28 has failed to evolve a more stable phenotype, or perhaps has evolved a less stable phenotype, because it is propagated in CAR-rich HeLa cells in a laboratory setting where it is not required to survive for very long at 37 °C in the absence of readily infected host cells.

In this report, we describe isolation of stable CVB3 strains from populations of CVB3/28 propagated under selective pressure, and characterization of the stabilizing effects of the individual capsid protein changes when introduced into CVB3/28. Co-infection of HeLa cells with CVB3/28 and a stable strain differing in a single capsid amino acid revealed a competitive advantage for the less stable CVB3/28.

RESULTS

CVB3/28CC, isolated previously from an RD cell carrier culture, was found to have a half-life of 13 h at 37 °C (S. D. Carson and N. M. Chapman, unpublished data; Table 1), and CVB3/28BS, isolated after seven cycles of CVB3/28 propagation in HeLa CCL-2 cells, had a half-life of 10 h at 37 °C (Table 1). These experiments showed that more stable CVB3/28 strains could be isolated from both RD cell carrier cultures and after serial propagation in HeLa CCL-2 cell cultures. In a subsequent experiment, CVB3/28 was propagated in HeLa CCL-2 cells, followed by a delay that required the virus to survive at 37 °C for 3–5 days in the absence of host cells before engaging the next infectious cycle (Fig. 1a). The first incubation (5 days at 37 °C) reduced the CVB3/28 titre from $1 \times 10^7$ to $2.5 \times 10^5$ TCID$_{50}$ ml$^{-1}$ (Fig. 1a). Virus stability increased with each of the subsequent seven selective cycles, after which the stability reached a plateau (Fig. 1b–d). The virus population that survived the 14th round of selection was designated CVB3/28N. Purified CVB3/28N diluted 1:9 into complete tissue culture medium had a half-life of 17 h at 37 °C (Fig. 2).

Sequence analysis of the CVB3/28N P1 region revealed three nucleotides that differed from the parental P1
sequence. These mutations resulted in two amino acid changes in Vp1 [glutamate to lysine at residue 80 and leucine to a mix of valine and leucine at residue 92]. The third mutation replaced alanine with threonine at residue 180 in Vp3. Lysine at Vp1 residue 80 was the sole position where the capsid of CVB3/28BS (t1/2 = 10 h) differed from CVB3/28, showing that this capsid mutation correlated with increased virus stability (possible mutations outside the capsid protein coding region were not investigated). To characterize the influence of the other two mutations identified in CVB3/28N with respect to virus stability, valine and threonine were introduced separately into CVB3/28 at Vp1 residue 92 and Vp3 residue 80, respectively. These strains were designated CVB3/28 1092V and CVB3/28 3180T, respectively. Analysis of virus decay at 37 °C showed that both CVB3/28 3180T (t1/2 = 10 h) and CVB3/28 1092V (t1/2 = 17 h) were more stable than CVB3/28 (t1/2 = 7 h), and that CVB3/28 1092V was as stable as CVB3/28N (Fig. 2). One-step growth curves (m.o.i. of 10 TCID50 per cell) showed that all three strains achieved maximum and equivalent infectious titre yields by 6 h after infection (Fig. 3).

Alignments of CVB3 Vp3 amino acid sequences revealed that all 45 CVB2, CVB4 and CVB6 sequences and 36/37 CVB5 sequences (numbering based on the CVB3/28 sequence) had valine at residue 180 (Table 2), whereas alanine occupied this position in all of the CVB1 sequences, and in 39/48 CVB3 sequences. Threonine was identified at Vp3 residue 180 in seven (15%) of the CVB3 sequences. Alignments of Vp1 sequences of the six coxsackievirus serotypes showed that threonine, glutamate and lysine were most common at residue 80, but these amino acids were highly segregated among serotypes (Table 2). Vp1 residue 80 was threonine in 98% of the CVB1 and CVB2 sequences. Among the CVB3 sequences, 4% had lysine at Vp1 residue 80 and glutamate was present in 94% of the sequences. One hundred out of 101 CVB4 sequences had glutamate at Vp1 residue 80. Lysine was found at Vp1 residue 80 in 98% of CVB5 and in all 46 CVB6 sequences. Analysis of 8138 enterovirus sequences found that isoleucine was highly conserved (96.5%) among all enteroviruses at residue 92 of Vp1. Valine was found at this position in 230 sequences (2.8%) and leucine in only 50 sequences (0.6%), 23 in the enterovirus B and 27 in the enterovirus D sequences. Among the enterovirus B species, of the 23 which had leucine at Vp1 residue 92, all were CVB3 and 18 were related to CVB3/Nancy.

We hypothesized that CVB3/28 remains less stable than other strains characterized with respect to stability at 37 °C because limited rounds of propagation in CAR-rich HeLa cells, as is customary using cloned infectious cDNA, does not preferentially select for more stable virus: there is no necessity to maintain a stable virion population when the virus receptor is present in abundance. When 2.4 × 10^6 HeLa cells were inoculated with 2.1 × 10^5 TCID50 of the 23 which had leucine at Vp1 residue 92, all were CVB3 and 18 were related to CVB3/Nancy of virus containing equal amounts of infectious CVB3/28 and CVB3/28 1092V, infectious virus continued to accumulate for over 50 h (Fig. 4a). Sequence analysis of Vp1 in these virus populations at sequential time points after inoculation revealed that the ratio of Leu:Val at residue 92, initially equal to 1, changed over time after inoculation (Fig. 4b). Leucine was almost twice as prevalent as valine after 8 h, but the ratio returned to unity at 12 h, consistent with the less stable CVB3/28 having completed its first round of replication faster than CVB3/28 1092V. Subsequent time points showed a saw-tooth pattern in the Leu:Val ratio, consistent with staggered replication cycles of the competing populations (Fig. 4b). From 10 h post-infection onwards, there was a slow increase in the Leu:Val ratio. Leucine exceeded valine at all points after 20 h, and the Leu:Val ratio versus time (Fig. 4b) had a positive slope, indicating a replicative advantage for the less stable phenotype. When the experiment was repeated with an m.o.i. of 30 TCID50 per cell, the shape of the one-step growth curves was repeated (Fig. 4c). The Leu:Val ratio at residue 92 in Vp1 was <1 at 2 h post-inoculation, but quickly rose above 3 by the end of the replication cycle (Fig. 4d). The fact that the Leu:Val ratio was <1 at 2 h post-inoculation suggests that the less stable CVB3/28 uncoated more rapidly than the more stable CVB3/28 1092V and sequestered limiting host factors, allowing it to dominate the post-replication population (Leu:Val ratio >3).

## DISCUSSION

Following the report that CVB3 strains propagated in a scarce-receptor cell culture environment were more stable than CVB3/28 (Carson et al., 2011), a simple question prompted the current study: why do all coxsackieviruses not evolve towards greater stability? The experiments intended to answer this question resulted in a second question: why is CVB3/28 atypically unstable?

We determined that CVB3/28 was capable of evolving to a more stable quasispecies phenotype when CVB3/28CC was isolated from an infected RD cell carrier culture and CVB3/28BS was isolated after seven rounds of propagation in HeLa CCL-2 cells. These results showed that a stable
CVB3/28 population could arise in environments that were CAR poor (RD cells) as well as CAR rich (HeLa cells). Note, however, that the serial propagation in HeLa CCL-2 cells had a selective component: the 48 h incubation required to complete the cytopathic effect in the HeLa CCL-2 cultures exceeded the CVB3/28 6 h replication cycle (Fig. 3) by 42 h. A single capsid mutation (glutamate to lysine at Vp1 residue 80) was present in the more stable CVB3/28BS. CVB3/28N was isolated after 14 rounds of intense selection for virus stability (Fig. 1). After the first round of propagation in HeLa CCL-2 cells and 37 °C incubation, the surviving CVB3/28A population had a mix of glutamate and lysine at residue 80 in Vp1, suggesting that this mutation is present in the CVB3/28 quasispecies after a single round of propagation in HeLa CCL-2 cells. After eight rounds of selection, CVB3/28H had a mix of alanine and threonine at Vp3 residue 180, glutamate and lysine at Vp1 residue 80, and leucine and valine at Vp1 residue 92. CVB3/28N, isolated after 14 rounds of selection, had threonine at Vp3 residue 180, lysine at Vp1 residue 80, and a mix of valine and leucine at Vp1 residue 92. CVB3/28 3180T and CVB3/28BS had intermediate stabilities, while valine at Vp1 residue 92 was individually capable of stabilizing the virus to the level achieved in CVB3/28N, i.e. the three mutations together

Fig. 1. (a) CVB3/28 was alternately propagated in HeLa CCL2 cells and allowed to decay at 37 °C for 17 cycles spanning 110 days. Post-selection virus substrains A–Q (●) were stored at −80 °C. TCID₅₀ ml⁻¹ was determined before (■) and after (●) the 37 °C incubation. Note the convergence plateau at about passage H. (b) Decay of CVB3/28A over time at 37 °C. The line represents the expected first-order decay curve for CVB3/28 (k=0.1 h⁻¹). (c) Decay curves for CVB3/28B, -C and -D (filled symbols) and CVB3/28E and -F (open symbols). (d) Decay curves for CVB3/28G to -Q. The curves in (c) were based on first-order decay of populations containing two virus strains with decay rates from the analyses in (b) and (d). Curves in (c) were fitted to the data by the method of least-squares (Levenberg–Marquardt) in PSI-Plot. The line in (d) was fitted by linear regression.
resulted in no greater stability than replacement of leucine by valine at Vp1 residue 92 alone.

Analysis of known CVB sequences revealed that alanine and valine predominated at Vp3 residue 180 in all CVB serotypes. Threonine has been reported in only CVB3, and in only seven of the 48 sequences, so this mutation is from a common amino acid to a relatively rare amino acid at this position. Similarly, glutamate was present at Vp1 residue 80 in 94% of CVB3 sequences and lysine in only 4%. Lysine dominates this position in CVB5 and CVB6, suggesting that the residue in this position may be favoured by pairing with other differences between serotypes. Nevertheless, this mutation in the more stable CVB3 was a shift from the common amino acid to a rare amino acid at this position. Surprisingly, leucine was found in Vp1 at residue 92 in only 4% of CVB3 sequences, most of which were related to CVB3/Nancy. Moreover, isoleucine, and valine to a lesser degree, dominated this position in Vp1 among all enteroviruses examined. The stabilizing mutation is from the very rare leucine at Vp1 residue 92 to a more common valine. This reveals that the CVB3/28 capsid is unusual among enteroviruses at Vp1 residue 92, and its short half-life relative to other CVB3 that have been characterized may be due to this rare variation.

It is informative to examine the position of these three mutations in the CVB3/M structure (Fig. 5, PDB 1COV; Muckelbauer et al., 1995). Vp1 residue 80 is located near the top of the fivefold prominence, where it is proximal to Vp1 residues 78 glutamate, 85 lysine, 86 arginine and 230 lysine. As far as can be discerned, the stabilizing glutamate to lysine mutation at Vp1 residue 80 is of interest insofar as glutamate may participate in ionic interaction with the three nearby positively charged side chains, while lysine will contribute to a positive charge cluster that may increase the capsid interaction with anionic secondary ligands (Carson et al., 2011; de Verdugo et al., 1995; Zautner et al., 2003, 2006). Vp3 residue 180 is inherently more interesting, for not only does it lie in the canyon that surrounds the fivefold axis of symmetry, but it is also in a Vp3 loop that is implicated in interaction with the ‘doorstop’ feature and that is displaced on capsid expansion in poliovirus (Strauss et al., 2015). In addition, while alanine at Vp3 residue 180 may have a hydrophobic interaction with the residue 146 proline in Vp1 of the adjacent protomer, threonine in this position has the added potential to contribute an additional hydrogen bond to the adjacent Vp1 backbone and thus strengthen inter-protomer binding. The stabilizing mutation at Vp3 residue 180 thus has the potential to affect receptor binding and capsid expansion.
Of the three mutations associated with increased virus stability, Vp1 residue 92 is the most interesting because it can individually determine whether the virus has the low stability of CVB3/28 or the high stability of CVB3/28N. Moreover, it lies in the hydrophobic pocket of Vp1 where the stabilizing lipid, called the pocket factor, and stabilizing antiviral compounds are bound (Lewis et al., 1998; Muckelbauer et al., 1995; Reisdorph et al., 2003; Tsang et al., 2000; van Vlijmen & Karplus, 2005). Leucine at Vp1 residue 92 has been shown to associate with CVB3 resistance to pleconaril (Groarke & Pevear, 1999; Schmidtke et al., 2005). Fig. 5, in which Vp1 residue 92 isoleucine in the 1COV structure has been replaced with leucine, illustrates that the leucine sidechain conflicts with the pocket factor, so either CVB3/28 cannot accommodate the pocket factor, the Vp1 pocket is conformationally different in CVB3/28 from the 1COV structure, or perhaps an alternative shorter pocket factor may be present, as has been described for EV-D68 and some less stable human rhinoviruses that have leucine side chains intruding into the pocket opposite Vp1 residue 92 (Liu et al., 2015). Considering the knowledge of pocket-binding molecules and the importance of the pocket in the conformational capsid breathing (Reisdorph et al., 2003), Vp1 residue 92 appears to be a key determinant of virus stability, and the rare leucine at this position among enteroviruses may

Table 2. Amino acid occurrence at Vp3 residue 180, Vp1 residue 80 and Vp1 residue 92 in CVB and Enterovirus sequences

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be sufficient to explain the unusually short half-life of CVB3/28. However, CVB3/RDVa has leucine at Vp1 residue 92 and is twice as stable as CVB3/28 (Carson et al., 2011), and both CVB3/28 3180T and CVB3/28BS are more stable than CVB3/28. Thus, there are compensatory mechanisms to stabilize these viruses even when Vp1 residue 92 is leucine.

Why do all group B coxsackieviruses not evolve towards greatest stability? The presence of leucine at Vp1 residue 92 predominately in CVB3 laboratory strains (related to Nancy), and in strains selected for resistance to antiviral drugs (Groarke & Pevear, 1999), indicates that a less stable virus is favoured when selection works against more stable virus populations. Such selection may be provided by the addition of drugs to the cultures, or by culture conditions that otherwise favour less stable viruses. The results of the experiments in which CVB3/28 competed against the more stable virus strain indicated that CVB3/28 uncoated more rapidly, and at high m.o.i. probably commandeered host factors required for replication. Even at a low m.o.i., the more unstable (quickly uncoating) virus, CVB3/28, accumulated over time relative to the more stable virus. This suggests that laboratory propagation of these viruses in receptor-rich cell cultures can select for the less stable variants in the quasispecies because they can uncoat more rapidly and

Fig. 4. Co-infection of HeLa cells with a low m.o.i. of CVB3/28 plus CVB3/28 1092Val (in equal amounts). (a) The strains were allowed to compete over multiple rounds of infection, and the TCID₅₀ ml⁻¹ increased over 50 h of culture. (b) After an initial early burst of CVB3/28 1092L, the ratio of Leu : Val returned to 1 after both strains completed a full round of replication. CVB3/28 then increased relative to CVB3/28 1092V from 18 to 50 h post-inoculation (H₀: slope=0 rejected at P<0.05 by Student’s t-test). The circles and squares in (a) and (b) represent results from two separate experiments. (c, d) When HeLa cells were infected with a high m.o.i. of CVB3/28 and CVB3/28 1092V, the TCID₅₀ ml⁻¹ recreated the single-step growth curves (c), but the Leu : Val ratio at each time point (d) indicated that CVB3/28 1092L uncoated more rapidly (Leu : Val ratio of <1 at 2 and 3 h) and generated over threefold more progeny by 8 h than CVB3/28 1092V.
commandeer the replication resources of the host cells. Conversely, when cells with sparse receptors are inoculated, the less stable virus populations decay disproportionately relative to more stable viruses before productive infection occurs, and the more stable virus population therefore has a selective advantage (Carson et al., 11). Such selection should initially be circumvented by the limited propagation of virus from cloned cDNA.

In nature, CVBs probably do evolve to an optimal stability, as evidenced by the preponderance of isoleucine at residue 92 in Vp1 among CVB and other enteroviruses. Enteroviruses spread predominantly by the faecal–oral route, a mode of transmission that requires them to survive transit through the gut, in faeces, at 37°C. In nature, CVBs probably do evolve to an optimal stability defined by their environment. This process is less than the maximum stability a capsid can achieve, for this is unnecessary, and is greater than that evinced by less stable (possibly attenuated for pathology; Groarke & Pevear, 1999) laboratory phenotypes, as this would place the population at a selective disadvantage. Our knowledge of less stable enterovirus virions may be the consequence of laboratory selection in receptor-rich cell culture environments.

METHODS

HeLa CCL-2 and RD CCL-136 cells were obtained from the American type Culture Collection. Laboratory strain HeLa cells (Lab HeLa) and RD3 cells (RD cells that express CAR) have been described elsewhere (Carson & Pirruccello, 2013; Cunningham et al., 2003). HeLa and RD lineages of these cell lines have been verified (Carson & Pirruccello, 2013). Cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM)-10 in a 37°C incubator 6% CO2. To prepare DMEM-10, each litre of DMEM (4.5 g glucose l−1) was supplemented with 100 ml FBS, 5 ml 200 mM glutamine, 10 ml penicillin/streptomycin (10000 U ml−1 and 10 mg ml−1, respectively) and 1.5 ml gentamicin (50 mg ml−1), all from Gibco /Life Technologies.

CVB3/28 (Tracy et al., 2002) was propagated following transfection of a cloned infectious cDNA in Lab HeLa cells. Virus stocks were prepared from frozen–thawed lysed monolayer cultures. Following removal of cell debris by low-speed centrifugation and extraction with chloroform, virus was partially purified by ultracentrifugation through 30% (w/v) sucrose, 1 mM MgCl2, 1 M NaCl and 0.02 M Tris/HCl (pH 7.5) into approximately 200 μl glycerol. Virus was resuspended in 0.1 M NaCl and stored at −80°C (Carson et al., 2011; Tu et al., 1995). Infectious virus titres were determined using RDt3 cells.

Strain CVB3/28BS was derived from CVB3/28 by serial passage in HeLa CCL-2 cells. The initial inoculation of 1.4 × 106 HeLa cells in a 25 cm2 flask used an m.o.i. of 14 TCID50 per cell. After 48 h, the medium was collected and cell debris was removed by centrifugation (1300 g for 10 min, at 4°C). An aliquot of the clarified supernatant (0.5 ml) was used to inoculate the next T25 flask of HeLa CCL-2 cells. The strain CVB3/28BS was collected after seven rounds of propagation, and the entire P1 (capsid protein) coding region sequence was determined (Carson et al., 2011).

The stable CVB3/28BN population was selected by serial CVB3/28 propagation in HeLa CCL-2 cells, with intervening periods of incubation at 37°C during which infectivity is lost (Organtini et al., 2014). Initially, HeLa CCL-2 cells at 80–90% confluency in 25 cm2 tissue culture flasks were inoculated with CVB3/28 at an m.o.i. of 20 TCID50 per cell. When the cytopathic effect was complete, or nearly so (typically 48 h; Fig. 1), the medium was removed, cleared of cell debris by centrifugation (1300 g for 10 min, at 4°C), placed into a new 25 cm2 flask and returned to the 37°C incubator. Three samples were taken from the cleared culture supernatant over the incubation period.
for determination of infectious titre and estimation of virus stability after each selective cycle (Fig. 1). At the end of each post-harvest incubation period, 2 ml supernatant was used to inoculate another flask of HeLa cells. The remaining supernatant was stored at ~80 °C. At the end of cycle 14, CVB3/28N was collected and the entire P1 (capsid protein) coding region sequence was determined (Carson et al., 2011). Virus stabilities were assessed in terms of the first-order rate constant for inactivation at 37 °C, as described previously in detail (Organtini et al., 2014). Stability was also expressed in terms of the virus infectious half-life (h) (t_{1/2} = \ln 0.5/−k, where k is the first order rate constant determined in Fig. 2).

In the competition experiments, the ratio of Leu:Val at residue 92 of VP1 was estimated by determining the thymine:guanine (T:G) ratio in the first position of the VP1 residue 92 codon from the sequence chromatograms. The ratio of T:G was determined as the ratio of the areas under the corresponding chromatogram peaks. Control sequences of CVB3/28 alone resulted in only T at this position, and sequences of the inoculum (equal infectious titres of each strain) resulted in a T:G ratio of 1.

Individual mutations were generated in the infectious CVB3/28 cDNA by overlap extension (Higuchi et al., 1988). Two cDNA fragments were generated using primers E6 (5’-CAGGATGTTGGCGTCGAGC-3’) and 1092Rev (containing a mutation altering the residue 92 codon of VP1 to express valine; 5’-CGTTGTTGATATCCCGATCCAGATCGGTTTGGG-3’) and primers 1092Fv for (containing a mutation altering the residue 92 codon of VP1 to express valine; 5’-GCTGATAGGG-TGCACCGACGACGAGC-3’) and XBA (5’-GCTGATCGTCTAAGTATCTGACCCGTGTTGAG-3’) in PCR with 10 U Deep Vent polymerase (New England Biolabs) ml−1 with primers at 0.5 μM, 200 mM dNTPs, 4 mM MgSO4 in 1 x Thermopol Buffer using 35 cycles of 94 °C for 30 s, 59 °C for 1 min and 72 °C for 2.5 min (E6/1092Fv) or 35 cycles of 94 °C for 30 s, 56 °C for 1 min and 72 °C for 3 min (VP1/1092Fv or XBA). The products of these PCRs were purified over columns (DNA Clean & Concentrate; Zymo Research), denatured, renatured and eluted with 10 U Deep Vent polymerase ml−1 with 200 mM dNTPs, 4 mM MgSO4 in 1 x Thermopol Buffer and 10 cycles of 94 °C for 1 min, 45 °C for 1 min and 72 °C for 5 min. Extended products were then amplified using 10 U Deep Vent polymerase ml−1 with primers E6 and XBA at 0.5 μM, 200 mM dNTPs and 4 mM MgSO4 in 1 x Thermopol Buffer using 35 cycles of 94 °C for 30 s, 57 °C for 30 s and 72 °C for 5 min. The cDNA product was then digested with restriction enzymes PsI and XbaI and ligated with a PsI/XbaI digest of a plasmid containing CVB3/28 cDNA with a deletion of the capsid region. Ligation were transformed into Escherichia coli DH5α, and plasmids with the incorporated cDNA were identified by the presence of a complete capsid sequence. This insert was sequenced completely to verify the presence of the VP1 residue 92 valine-encoding mutation.וך

The human enterovirus A–D species protein sequences (from GenBank) with significant similarity to each species reference protein sequence of VP1 were aligned using BLAST (Altschul et al., 2005). By alignment with the CVB3/28 VP1 sequence, sites corresponding to amino acids of CVB3/28 were located. This approach was also used to align VP3 sequences of the six CVB serotypes.

REFERENCES


