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Short communication

New recognition of *Enterovirus* infections in bottlenose dolphins (*Tursiops truncatus*)

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ABSTRACT

An enterovirus was cultured from an erosive tongue lesion of a bottlenose dolphin (*Tursiops truncatus*). The morphology of virions on negative staining electron microscopy was consistent with those of enteroviruses. Analysis of 2613 bp of the polyprotein gene identified the isolate as a novel enterovirus strain, tentatively named bottlenose dolphin enterovirus (BDEV), that nests within the species *Bovine enterovirus*. Serologic evidence of exposure to enteroviruses was common in both free-ranging and managed collection dolphins. Managed collection dolphins were more likely to have high antibody levels, although the highest levels were reported in free-ranging populations. Associations between enterovirus antibody levels, and age, sex, complete blood counts, and clinical serum biochemistries were explored. Dolphins with higher antibody levels were more likely to be hyperproteinemic and hyperglobulinemic.

1. Introduction

The enteroviruses (EV) are members of the genus *Enterovirus* within the family *Picornaviridae*. Enteroviruses were originally distinguished from these other picornaviruses by their physical, chemical and antigenic characteristics. Current state-of-the-art virus identification and characterization of EV consist largely of sequencing of the viral genome and pairwise genomic sequence alignments. The recognized members of the genus *Enterovirus* consist of the human enteroviruses A–D, poliovirus, porcine enteroviruses A and B, Simian enterovirus A, and bovine enteroviruses A and B (Stanway et al., 2005; Zell et al., 2006). The EV genome contains only one open reading frame (ORF) encoding a large polyprotein which is subsequently cleaved to give the various viral proteins. Genetic distance analyses using the immunodominant VP1 capsid coding region of the EV genome appear to mirror the antigenic relatedness more closely (Oberste et al., 1999). It is commonly accepted that VP1 nucleotide identities of greater than 75% suggest that isolates are serologically identical.

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Most EV enter the host via the oral route and establish infections in the small intestine. EV can be recovered in the acute phase of the infection from the saliva and throat swabs. Most EV infections do not appear to cause clinical disease, but associated clinical signs can include fever, gastrointestinal disease, meningitis, myocarditis, inflammatory myopathies, abortion, and mucocutaneous blisters (Pallansch and Roos, 2007). High levels of neutralizing antibodies are typically generated following EV infection, which usually result in life-long immunity to clinical disease (Minor et al., 1981). This robust antibody response is, in part, responsible for the high prevalence of EV-specific antibodies in the human population (Melnick, 1996).

Here, we report on the isolation and characterization of an enterovirus isolated from an erosive tongue lesion in an adult female Atlantic bottlenose dolphin (*Tursiops truncatus*). The amino-terminal portion of the predicted viral polyprotein was used to determine the phylogenetic classification of the bottlenose dolphin enterovirus (BDEV). The prevalence and clinical relevance of BDEV-like infections in dolphins were assessed in a retrospective serologic survey of wild, stranded, and managed collection dolphin populations.

2. Materials and methods

2.1. Virus isolation

The case dolphin was a 16-year-old, clinically healthy, female Atlantic bottlenose dolphin that presented with multifocal, small (approximately 1 mm), and erosive tongue lesions. The dolphin was part of a managed collection and was housed in a coastal open ocean water enclosure. No behavioral abnormalities were noted. A throat swab was collected from the case dolphin using a sterile cotton swab and was mailed overnight on ice to a reference laboratory for viral isolation using African green monkey kidney (Vero) cells (Supplemental Materials). One infected Vero cell monolayer exhibiting CPE was processed for negative staining electron microscopy (NEM) (Fig. 1 and Supplemental Materials).

2.2. Molecular characterization and phylogeny

A viral genomic sequence segment was generated through degenerate PCR (Nix et al., 2006). The viral isolate was also studied by using GreeneChip Vr1.5 (Palacios et al., 2007). Hybridized cDNA was eluted from the microarray and was sequenced. The contiguous sequence was obtained by primer walking between these sequences (Fig. 2 and Supplemental Materials: Table 1). Each nucleotide position was sequenced at least three times in each direction. Sequence identity was determined via pairwise comparison with the sequences in GenBank, EMBL and the Data Bank of Japan using TBLASTX (Altschul et al., 1997). Phylogenetic placement was determined for the predicted VP1, VP2, and VP3 proteins individually using maximum likelihood and Bayesian posterior analysis (Fig. 3 and Supplemental Materials).

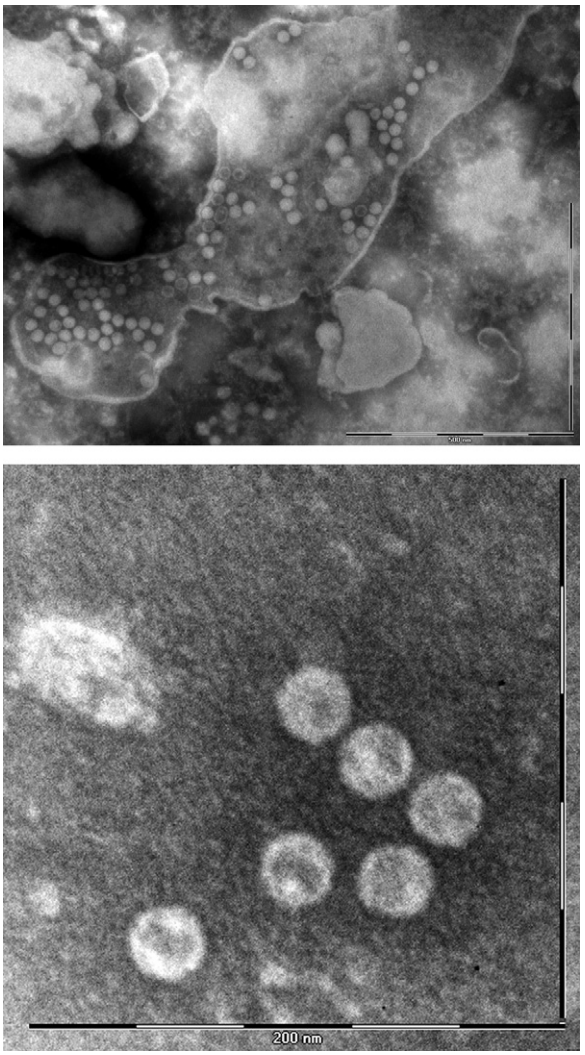

Fig. 1. Negatively stained preparations of the bottlenose dolphin enterovirus BDEV as seen by electron microscopy. Bar = 500 nm (top) and 200 nm (bottom). The size and morphology of the virions were consistent with those of enteroviruses. Empty capsids were occasionally observed.

2.3. ELISA development

Purified BDEV was used as antigen in the whole virus-based indirect ELISA system (Supplemental Materials). The dolphin sera were analyzed in triplicate and a no-serum negative control and one reference serum sample were included on each plate. A biotinylated monoclonal antibody specific for bottlenose dolphin IgG (Nollens et al., 2007) was used for the detection of bound antibodies. The OD_{405} was recorded 60 min after addition of the substrate. For analysis, all results were presented as OD_{405} ratios, defined as the mean OD_{405} of the triplicate readings of the unknown samples divided by the mean OD_{405} reading of the reference sample.

2.4. Serum sample collection

Serum samples were collected from five dolphin populations (Supplemental Materials: Table 2). Population

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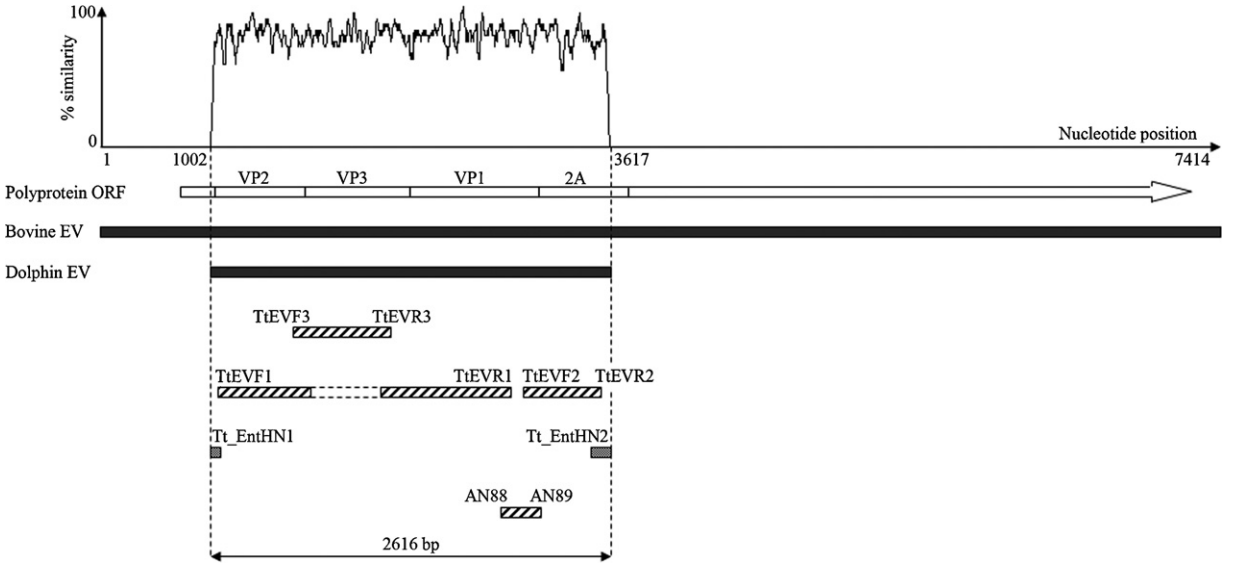

Fig. 2. Diagram representing the percent nucleotide similarity (calculated using 20 bp windows) and the alignment of the 2616 bp BDEV genome segment compared to the full-length genome sequence of bovine enterovirus strain BEVVG527 (7414 bp, GenBank accession # D00214). The position of the ORF encoding the polyprotein is shown using the box arrow and the polyprotein cleavage sites for the viral peptides VP2, VP3, VP1 and 2A are indicated. The relative position of the genome segments generated using degenerate PCR (vertical stripes), GreeneChip viral microarray (shaded) and specific PCRs (diagonal stripes) are indicated.

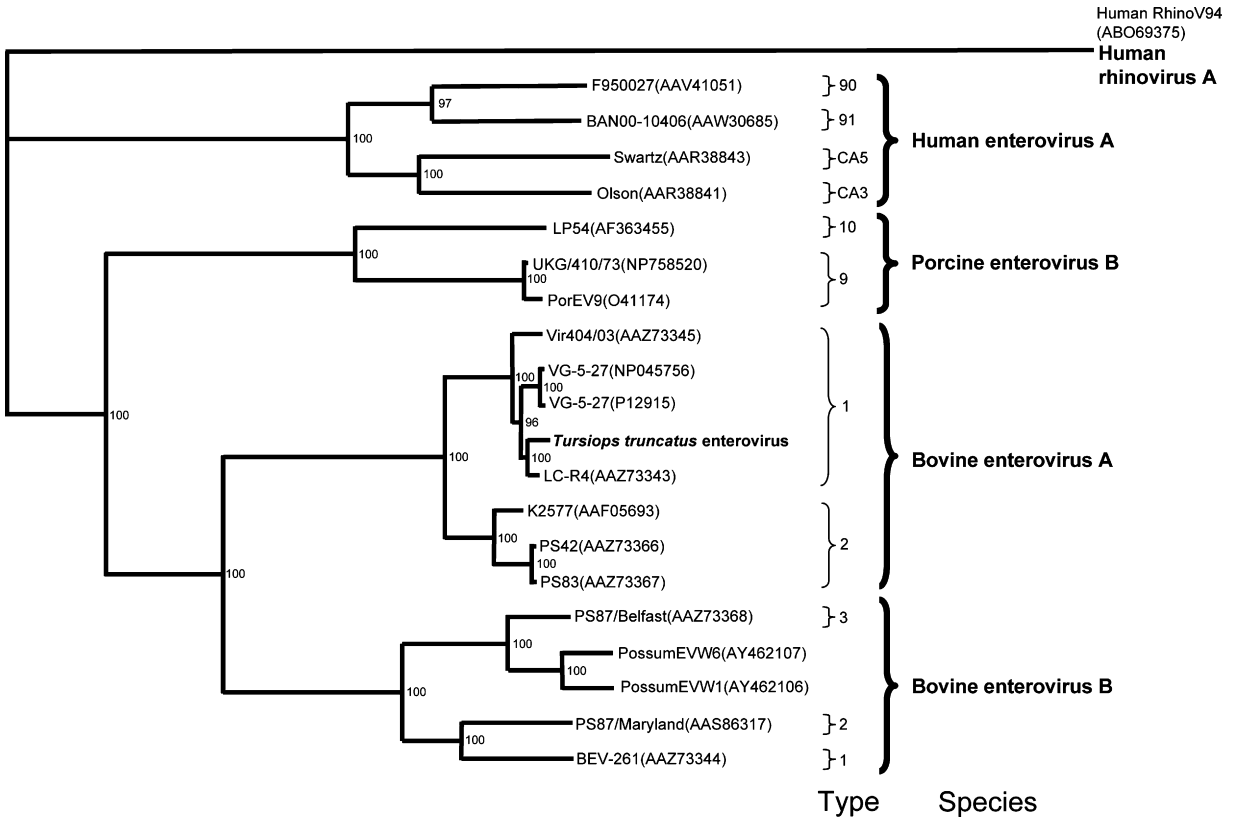

Fig. 3. Bayesian phylogenetic tree of predicted 864–897 amino acid sequences of BDEV and other EV based on MUSCLE alignment. Bayesian posterior probabilities of branchings are given as percentages, Human rhinovirus 94 (GenBank accession number ABO69375) was designated as the outgroup. Virus types are delineated by brackets, and virus species are delineated by heavy brackets. *Bottlenose dolphin* enterovirus is given in bold.

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A represents clinically healthy, free-ranging dolphins from Florida ($N = 58$), whereas population B represented ill, stranded dolphins from Florida ($N = 7$). Populations C ($N = 52$) and D ($N = 21$) represented two managed collections housed in land-based closed systems. Population E ($N = 63$) was the dolphin population, housed in coastal ocean pens from which the case dolphin originated. Full serum chemistry panels and complete blood counts were available for population E only.

2.5. Data analysis

2.5.1. Population seroprevalence

Because of the lack of control samples and the resulting inability to calculate a positive:negative cut-off value, a conservative and relational interpretation of ELISA results was adopted. Mean, standard deviation, median, and range of OD_{405} ratios were compared among all five dolphin populations. OD_{405} ratios greater than the 75th percentile were defined as 'high' (>1.1), less than the 25th percentile were defined as 'low' (<0.7), and between the 25th and 75th percentiles as 'moderate' (0.7–1.1). An analysis of variance was conducted to compare mean EV antibody OD_{405} ratios by population (Supplemental Materials: Table 2), and box plots were created to assess OD_{405} ratio distributions by population (Fig. 4). A χ^2 test was run to test differences in the prevalence of low, moderate, and high EV antibody OD_{405} ratios by population; a Mantel–Haenszel χ^2 P value was used to determine significance (Fig. 5). In both the ANOVA and χ^2 test, wild stranded data were excluded due to the low number of animals ($N = 7$).

2.5.2. Risk assessment and clinical relevance

Age, sex, complete blood cell (CBC) counts, and serum clinical chemistry data from population E from clinically healthy and ill animals during January 2005 through November 2006, were stratified using simple linear regression and analyses of covariance (ANCOVA) to determine the potential influence of age, sex, and clinical diagnostic values on OD_{405} ratios. The model controlled for age, sex, and fasting status, which can affect blood parameters (Venn-Watson et al., 2007). Least squares means were reported and type I sum of squares P value was used. A post hoc chi-square analysis was conducted on variables significantly associated with OD_{405} ratios to compare high and not high serum protein (high >7.6 g dL^{-1}), albumin (high >4.8 g dL^{-1}), globulins (high >3.1 g dL^{-1}), and reason for blood collection (routine or clinical sample). Due to low numbers in some chi-square categories, a Fisher's exact test P value was used to determine significance.

P values ≤0.01 were defined as significant for all statistical tests except for chi-square analyses involving low sample numbers, in which a P value <0.05 was considered significant.

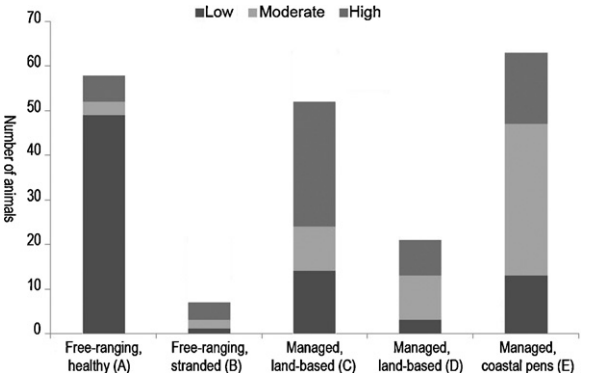

Fig. 5. Comparisons of number of bottlenose dolphins with low, moderate, and high enterovirus OD_{405} ratios, by population type ($P = 0.01$). Free-ranging, stranded dolphins were not included in the comparative analysis due to a low N (7).

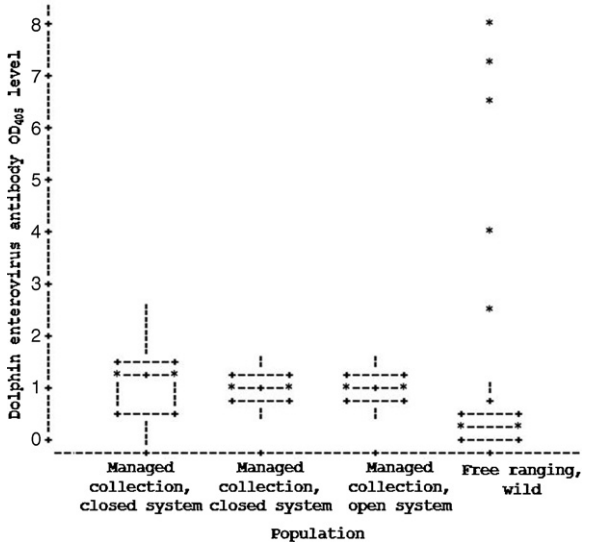

Fig. 4. Box plots of enterovirus OD_{405} ratio distributions, by bottlenose dolphin population type. Free-ranging, stranded dolphins were not included in the comparative analysis due to a low N (7).

3. Results

3.1. Virus isolation

Cytopathic effects suggestive of a virus growth (cell lysis and plaque formation) were observed after 6 days. NEM of the infected monolayers revealed monomorphic small viral particles of 25–30 nm in diameter that lacked a lipid membrane (Fig. 1).

3.2. Molecular characterization and phylogeny

A contiguous molecule of 2613 bp was generated (GenBank # EU886967) via degenerate PCR, panviral microarray and primer walking. The molecule was homologous to the 1002–3617 bp region of the BEVVG527 genome (Fig. 2), which comprises the region encoding VP2, VP3 and VP1. TBLASTX results for the full sequence determined showed the highest score with *Bovine enterovirus* strain LC-R4 (GenBank # DQ092769). BLASTP results for the VP1 and VP2 predicted amino acid sequence showed the highest score with *Bovine enterovirus A* type 1 strains Vg-5-27 (GenBank # 1BEV_1) Vir 404/03 (GenBank

AAZ73345) with at least 95% amino acid identity. VP1 genes of the same bovine enterovirus serotypes have >90% amino acid identity (Zell et al., 2006). BDEV was strongly supported to be in the clade designated as *Bovine enterovirus A* (BEVA) type 1 (Fig. 3; Zell et al., 2006), with a posterior probability of 1.000.

3.3. Population seroprevalence

Mean EV antibody OD_{405} ratios were not different among free-ranging, stranded and managed collection dolphins ($P = 0.3$). Mean OD_{405} ratios ranged from 0.8 to 1.3, with the lowest OD_{405} ratio measured in free-ranging dolphins and the highest OD_{405} ratio measured in stranded dolphins (Supplemental Materials: Table 2). The highest values were reported in stranded and free-ranging populations, although managed collection dolphins were more likely to have high OD_{405} ratios ($P = 0.01$).

3.4. Risk assessment and clinical relevance

Subjects included in this part of the study consisted of 63 dolphins aged 0.2–49.2 years. Nine (14.3%) of the 63 animals had blood drawn for clinical reasons, and the remaining samples were collected for routine purposes. Neither age ($P = 0.2$) nor sex was significant predictor of EV antibody OD_{405} ratios (mean OD_{405} ratio, females = 0.9 and males = 1.0, $P = 0.2$). The prevalence of low, moderate, or high OD_{405} ratios were not different between clinical and routine samples (% high OD_{405} ratios = 33% and 24%, respectively; $P = 0.81$).

Of 30 blood panel variables, only total serum protein level was significantly associated with OD_{405} ratio (Supplemental Materials: Table 3). Specifically, dolphins with high EV antibody OD_{405} ratios were more likely to have higher protein compared to dolphins with moderate or low EV OD_{405} ratios (serum protein = 7.3, 7.0 and 6.6 g dL^{-1}, respectively; $P = 0.006$). Further, dolphins with high EV OD_{405} ratios were eight times more likely to have hyperproteinemia (serum protein >7.6 g dL^{-1}) and 5.9 times more likely to have hyperglobulinemia (serum globulins >3.1 g dL^{-1}) compared to dolphins with moderate to low OD_{405} ratios ($P = 0.02$ and $P = 0.04$, respectively). No significant differences were identified when comparing OD_{405} ratios among dolphins with high or normal serum albumin levels ($P = 0.4$).

4. Discussion

The virus isolate in this report, which was named BDEV, is the first reported enterovirus and only the second reported picornavirus in a marine mammal (Kapoor et al., 2008). The virus isolated in this study was morphologically and molecularly identified as *Bovine enterovirus A*. Since the predicted VP1 amino acid sequence was 95% identical to the VP1 sequence of BEVA type 1, BDEV should be considered as a novel strain within the BEVA type 1 complex (Zell et al., 2006).

BDEV is likely of recent bovine origin. Related species such as domestic cattle and dolphins will have similar host proteins and receptors, and thus viruses may be able to cross to closely related host species more easily. A parainfluenza virus of dolphins is most related to and likely evolved from bovine parainfluenza virus 3 (Nollens et al., 2007). However, unlike TtPIV-1, BDEV has not diverged significantly from the corresponding bovine virus, suggesting very recent marine–terrestrial transmission. Bovine enteroviruses have previously demonstrated the potential for host-switching. It is suspected that swine vesicular disease virus was introduced from humans into pigs (Zhang et al., 1992), and two enteroviruses isolated from brushtail possums form a clade that clusters with the BEVB (Zheng, 2007).

It is difficult to obtain unequivocally negative serum samples from wildlife species (Nollens et al., 2006; Venn-Watson et al., 2008). In the absence of sufficient unequivocal control sera we were unable to determine a positive:negative cut-off value for the assay. Instead of categorizing samples as positive or negative, ELISA results were expressed as ratios to a reference serum sample. ELISA data were then used to help clarify the clinical relevance and relative seroprevalence among five dolphin populations housed under different conditions. BDEV was isolated from erosive oral lesions from a bottlenose dolphin. The clinical presentation in the case dolphin resembles enteroviral hand, foot and mouth disease. However, a larger-scale risk assessment of BDEV was unable to associate exposure to BDEV with changes in hematological parameters or outward clinical signs. The role and relevance of hyperglobulinemia in animals with high EV antibody levels are not understood and may not be clinically relevant. While continued monitoring may identify pathologies associated with BDEV, our findings so far suggest that BDEV infections in bottlenose dolphins, like those in several other species, are largely subclinical. In humans, shedding of EV does not necessarily indicate disease, because most infections and subsequent shedding are asymptomatic (Morens et al., 1979; Moore, 1982).

The mean exposure status of the free-ranging and managed populations was not different among five dolphin populations. Several cross-reactive EV types typically co-circulate in humans (Morens et al., 1979; Moore, 1982). Since this is the only known dolphin EV type, we are unable to determine specificity of the assay and cannot evaluate heterotypic responses that have been reported in humans (Swanink et al., 1993). Since the ELISA is a whole virus-based assay, it may be appropriate to assume heterotypic responses with other, unidentified dolphin EV types do occur. The serologic data, both within and between dolphin populations, may therefore reflect cumulative incidence and prevalence of several distinct dolphin EV types.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.vetmic.2009.05.010.

References

Altschul, S.F., Madden, T.L., Schäffer, A.A., Zhang, J., Zhang, Z., Miller, W., 1997. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res. 25, 3389–3402.

Kapoor, A., Victoria, J., Simmonds, P., Wang, C., Shafer, R.W., Nims, R., Nielsen, O., Delwart, E., 2008. A highly divergent picornavirus in a marine mammal. J. Virol. 82, 311–320.

Minor, T.E., Allen, C.I., Tsiatis, A.A., Nelson, D.B., D'Alessio, D.J., 1981. Human infective dose determinations for oral poliovirus type 1 vaccine in infants. J. Clin. Microbiol. 13, 388–389.

Melnick, J.L., 1996. Current status of poliovirus infections. Clin. Microbiol. Rev. 9, 293–300.

Moore, M., 1982. Centers for Disease Control. Enteroviral disease in the United States, 1970–1979. J. Infect. Dis. 146, 103–108.

Morens, D.M., Zweighaft, R.M., Bryan, J.M., 1979. Non-polio enterovirus disease in the United States, 1971–1975. Int. J. Epidemiol. 8, 49–54.

Nix, W.A., Oberste, M.S., Pallansch, M.A., 2006. Sensitive, seminested PCR amplification of VP1 sequences for direct identification of all enterovirus serotypes from original clinical specimens. J. Clin. Microbiol. 44, 2698–2704.

Nollens, H.H., Gulland, F.M.D., Hernandez, J.A., Condit, R.C., Klein, P.A., Walsh, M.T., Jacobson, E.R., 2006. Seroepidemiology of poxvirus infections in captive and free-ranging California sea lions (*Zalophus californianus*). Dis. Aquat. Org. 69, 153–161.

Nollens, H.H., Wellehan, J.X., Saliki, J.T., Caseltine, S.L., Jensen, E.D., Van Bonn, W., Venn-Watson, S., 2007. Characterization of a parainfluenza virus isolated from a bottlenose dolphin (*Tursiops truncatus*). Vet. Microbiol. 128, 231–242.

Oberste, M.S., Maher, K., Kilpatrick, D.R., Pallansch, M.A., 1999. Molecular evolution of the human enteroviruses: correlation of serotype with VP1 sequence and application to picornavirus classification. J. Virol. 73, 1941–1948.

Palacios, G., Quan, P.L., Jabado, O.J., Conlan, S., Hirschberg, D.L., Liu, Y., 2007. Panmicrobial oligonucleotide array for diagnosis of infectious diseases. Emerg. Infect. Dis. 13, 73–81.

Pallansch, M., Roos, R., 2007. Enteroviruses. In: Knipe, D.M. (Ed.), Fields Virology. 5th ed. Lippincott Williams and Wolters Kluwer Business, Philadelphia, pp. 839–893.

Stanway, G., Brown, F., Christian, P., Hovi, T., Hyypiä, T., King, A.M.Q., Knowles, N.J., Lemon, S.M., Minor, P.D., Pallansch, M.A., Palmenberg, A.C., Skern, T., 2005. Picornaviridae. In: Fauquet, C.M., Mayo, M.A., Maniloff, J., Desselberger, E., Ball, L.A. (Eds.), Virus Taxonomy: Classification and Nomenclature of viruses. Eighth Report of the International Committee on the Taxonomy of Viruses. Academic Press, San Diego, CA, pp. 757–778.

Swanink, C.M., Veenstra, L., Poort, Y.A., Kaan, J.A., Galama, J.M., 1993. Coxsackievirus B1-based antibody-capture enzyme-linked immunosorbent assay for detection of immunoglobulin G (IgG), IgM and IgA with broad specificity for enteroviruses. J. Clin. Microbiol. 31, 3240–3246.

Venn-Watson, S., Jensen, E.D., Ridgway, S.H., 2007. Effects of age and sex on clinicopathologic reference ranges in a healthy managed Atlantic bottlenose dolphin population. J. Am. Vet. Assoc. 231, 596–601.

Venn-Watson, S., Rivera, R., Smith, C.R., Saliki, J.T., Caseltine, S., St. Leger, J., Yochem, P., Wells, R., Nollens, H.H., 2008. Exposure to novel parainfluenza virus and clinical relevance in 2 bottlenose dolphin (*Tursiops truncatus*) populations. EID 14, 397–405.

Zell, R., Krumbholz, A., Dauber, M., Hoey, E., Wutzler, P., 2006. Molecular-based reclassification of the bovine enteroviruses. J. Gen. Virol. 87, 375–385.

Zhang, G., Haydon, D.T., Knowles Nj, McCauley, J.W., 1992. Molecular evolution of swine vesicular disease virus. J. Gen. Virol. 80, 639–651.

Zheng, T., 2007. Characterisation of two enteroviruses isolated from Australian brushtail possums (*Trichosurus vulpecula*) in New Zealand. Arch. Virol. 152, 191–198.

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