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Genotypes of Pestivirus RNA Detected in Live Virus Vaccines for Human Use

Massimo GIANGASPERO[1)], Giovanni VACIRCA[1)], Ryô HARASAWA[2)]*, Mathias BÜTTNER[3)], Alfonso PANUCCIO[4)], Carlo DE GIULI MORGHEN[5)], Alessandro ZANETTI[6)], Angelo BELLOLI[1)] and Antoine VERHULST[7)]

[1)]*Institute of Special Pathology and Veterinary Medical Clinic, Faculty of Veterinary Medicine, The University of Milan, Milan, Italy,* [2)]*Animal Center for Biomedical Research, Graduate School of Medicine, The University of Tokyo, Tokyo, Japan,* [3)]*Institute of Immunology, Federal Research Centre for Virus Diseases of Animals, Tübingen, Germany,* [4)]*Multizonal Center for Hygiene and Prevention, Milan, Italy,* [5)]*Institute of Pharmacology, Faculty of Medicine, The University of Milan, Milan, Italy,* [6)]*Institute of Virology, Faculty of Medicine, The University of Milan, Milan, Italy and* [7)]*Prince Léopold Institute of Tropical Medicine, Antwerp, Belgium*

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ABSTRACT. Live virus vaccines for human use, 29 monovalent vaccines against measles, mumps, rubella or polio, eight polyvalent vaccines against measles-mumps-rubella and one bacterial polyvalent vaccine against *Streptococcus pneumoniae*, were tested by reverse transcriptase-nested PCR for the presence of petivirus or pestivirus RNA. Twenty-four samples were selected from European manufacturers, ten were from U.S.A. and four from Japan. Five (13.1%) out of 38 tested samples were positive for pestivirus RNA. Three vaccines (rubella and two measles) were from Europe and two (mumps and rubella) from Japan. The 5'-untranslated genomic region of the contaminant pestivirus RNA were amplified by reverse transcription-PCR and sequenced. Analyses based on primary nucleotide sequence homology and on secondary structures, characteristic to genotypes, revealed that the cDNA sequences belonged to bovine viral diarrhea virus (BVDV). A cDNA sequence, detected from one measles sample, belonged to BVDV-1b genotype. Pestiviral cDNA detected from the Japanese mumps and rubella vaccine samples, belonged to the BVDV genotypes 1a and 1c, respectively. Analysis on two cDNA sequences detected from measles and rubella vaccine samples from Europe showed their appurtenance to a new genotype, BVDV-1d. These findings indicate that contamination by animal pestivirus may occur in biological products for human use.
KEY WORDS: contamination, genotype, pestivirus, vaccine.

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Bovine viral diarrhea virus 1 (BVDV-1), *Bovine viral diarrhea virus 2* (BVDV-2), *Border disease virus* (BDV) and *Classical swine fever virus* (CSFV) are species of the genus Pestivirus of the family *Flaviviridae* [44], and they are cosmopolitan pathogens in cloven-footed ungulates.

According to the classification proposed by Pellerin *et al.* [32] and further applications [19, 20], based on the nucleotide divergence in the 5'-untranslated region (UTR) of genomic RNA, the BVDV-1 can be segregated into at least three genotypes, BVDV-1a, BVDV-1b and BVDV-1c. Genotypes BVDV-1a and BVDV-1b represent the majority of classical BVDV strains such as NADL, SD-1, Oregon, Singer, Osloss and NY-1 reference strains. Strain Europa, a non-cytopathic (NCP) BVDV strain, isolated from a human buffy coat sample [16, 17, 19] and three bovine field isolates [49] were classified as the BVDV-1c genotype. BVDV-2, which was first found in a human virus vaccine [24], includes the reference strains 890 and CD87 isolated from outbreaks characterized by thrombocytopenia and high mortality of cattle in U.S.A. and Canada [32, 36].

A relevant and common aspect is the occurrence of contamination of biologicals, cell cultures including primary cell cultures and cell lines, even of human origin [5, 7, 22, 25, 33], fetal bovine serum (FBS) [3, 4, 31] and vaccines for veterinary use [26, 28, 43, 47] by NCP strains of BVDV. Recently, serious BVD outbreaks in Dutch cattle farms were reported due to vaccine contamination by BVDV-2 [13].

During an experimental study in Japan, pestiviral RNA was also detected in live virus vaccines for human use [24, 40]. The vaccines were produced by different pharmaceutical companies, regularly authorized and commercialized: two monovalents against mumps and rubella and two polyvalents against measles, mumps and rubella. Comparative analysis of the nucleotide sequence at the 5'-UTR identified BVDV RNA as the contaminant. Similarly, interferon for human use was found contaminated by BVDV RNA [23]. Recently, Vilcek *et al.* [46] reported negative results from 30 human virus vaccines produced by European manufacturers screened by PCR for pestivirus or pestiviral RNA. They concluded that the occurrence of human virus vaccines contamination does not represent a wide-spread phenomenon.

Giving the importance of safety of biological products for human use, the present study was undertaken with the aim to provide confirmation of the previous reports from Japan and verify the findings reported by Vilcek *et al.* [46] in order to evaluate pestiviral RNA contamination in live virus vaccines for human use.

MATERIALS AND METHODS

Vaccine samples: Tests were performed on 38 virus vaccine samples for human use, from 13 lots, produced by six different manufacturers (mentioned here from A to F) from Europe, Japan and U.S.A. (Table 1). Thirty-seven contained modified live virus antigens, one was a bacterial inac-

* CORRESPONDENCE TO: DR. HARASAWA, R., Animal Center for Biomedical Research, Graduate School of Medicine, The University of Tokyo, Bunkyo-ku, Hongo 7–3–1, Tokyo 113–0033, Japan.

Table 1. Live virus vaccines for human use tested for pestiviral RNA by nested PCR

No	Virus antigen	Cell substrate	Lot	Manufacturer	Origin country
1	measles	human diploid	1	A	Switzerland
2	measles	human diploid	1	A	Switzerland
3	MMR	human diploid	2	A	Switzerland
4	MMR	human diploid	3	B	USA
5	rubella	human diploid	4	C	Italy
6	rubella	human diploid	4	C	Italy
7	measles	chick fibroblast	5	D	Japan
8	rubella	quail fibroblast	6	D	Japan
9	rubella	rabbit kidney	7	E	Japan
10	mumps	chick fibroblast	8	E	Japan
11	measles	human diploid	1	A	Switzerland
12	measles	human diploid	1	A	Switzerland
13	measles	human diploid	1	A	Switzerland
14	measles	human diploid	1	A	Switzerland
15	measles	human diploid	1	A	Switzerland
16	MMR	human diploid	3	B	USA
17	MMR	human diploid	3	B	USA
18	MMR	human diploid	3	B	USA
19	MMR	human diploid	3	B	USA
20	MMR	human diploid	3	B	USA
21	MMR	human diploid	9	B	USA
22	rubella	human diploid	4	C	Italy
23	rubella	human diploid	4	C	Italy
24	rubella	human diploid	4	C	Italy
25	rubella	human diploid	4	C	Italy
26	rubella	human diploid	4	C	Italy
27	polio	monkey kidney	10	C	Italy
28	polio	monkey kidney	11	C	Italy
29	polio	monkey kidney	10	C	Italy
30	polio	monkey kidney	10	C	Italy
31	polio	monkey kidney	10	C	Italy
32	polio	monkey kidney	10	C	Italy
33	polio	monkey kidney	12	F	Belgium
34	polio	monkey kidney	12	F	Belgium
35	polio	monkey kidney	12	F	Belgium
36	polio	monkey kidney	12	F	Belgium
37	polio	monkey kidney	12	F	Belgium
38	Str. pneumoniae*		13	B	USA

*: Bacterial vaccine.

tivated vaccine. In three assays BVDV reference strains Oregon, NY-1 and NADL were used as a positive control. Samples 1, 3, 5, 13, 18, 24, 30 and 35 were tested in a Belgian laboratory, samples 2, 4, 6, 12, 17, 23, 29 and 34 were tested in Germany, samples 7, 8, 9, 10, 11, 16, 21, 22, 27, 28, 33 and 38 in Japan, samples 14, 19, 25, 31 and 36, 15, 20, 26, 32 and 37 in two different Italian laboratories. Samples were stored at –70°C until examination.

RNA extraction: Viral RNA was extracted from each sample by the single-step guanidinium isothiocyanate-phenol-chloroform method [8] with RNAzol B extraction kit (Biotecx Laboratories Inc., U.S.A.) or TRIZOL (Gibco BRL, Md., U.S.A.). For the RNAzol B extraction, 200 μl of each sample were mixed vigorously with 800 μl of RNAzol B in a 1.5-m*l* Eppendorf tube for 30 sec. One hundred microliters of chloroform were added and the tube was vigorously shaken by hand for 30 sec. The tube was chilled in ice for 5 min and centrifuged at 12,000 rpm for 10 min. The aqueous phase (ca. 600 μl of supernatant fluid) was transferred into a fresh Eppendorf tube. One microliter of 20 mg/m*l* mussel glycogen (Boehringer Mannheim GmbH, Germany) and 600 μl of isopropanol were added and the tube was chilled in ice for 30 min. The RNA precipitate was collected by centrifugation at 12,000 rpm for 10 min. The pellet was washed three times with 600 μl of 75% ethanol in water treated with 0.1% diethyl pyrocarbonate (DEPC). The pellet was air-dried at 37°C for 10 min, dissolved in 20 μl of distilled water treated with 0.1% DEPC, and then heated at 60°C for 10 min. For the TRIZOL extraction, each sample was mixed with 1 m*l* of TRIZOL and incubated at room temperature for 5 min. Two hundred microliters of chloroform were added and the tube was vigorously shaken and incubated at room temperature for 3 min. The solution was centrifuged at 12,000 rpm at 4°C for 15 min. The aqueous phase was collected and 500 μl of isopropanol were added and the tube was centrifuged at 12,000 rpm at 4°C for

10 min. The pellet was washed three times with 1 m*l* of 75% ethanol and centrifuged again at 12,000 rpm at 4°C for 15 min. The pellet was air-dried at 37°C for 5 min, dissolved in 10 μl of distilled water treated with 0.1% DEPC. The RNA solution (16 μl) was subjected to reverse transcription (RT) reaction.

Oligonucleotides: Oligonucleotide primer sequences of the 5'-UTR used for RT-PCR are same as described previously [25], which are based on genomic sequences of high homology among those described by Collett *et al.* [9], De Moerlooze *et al.* [10], Deng and Brock [11], Meyers *et al.* [29] and Roehe *et al.* [38]. These primer sequences are not found in any other viral sequences published so far. Primers 5'-TCAACTCCATGTGCCATGTAC-3' for reverse (R), 5'-CATGCCCTTAGTAGGACTAGC-3' for forward (F), 5'-CTCTGCAGCACCCTATCA-3' for R2 and 5'-AGGGTAGTCGTCAGTGGTTCG-3' for F2 were used to test samples 1, 3, 5, 7, 13, 18, 24, 30 and 35. Their equivalent positions in the NADL strain of BVDV-1 are 107-127 for F, 373-395 for R, 185-205 for F2 and 326-343 for R2, respectively. Primers 5'-ATGCCC(A/T)(C/T)AGTAGGACTAGC-3' for forward (F), 5'-ACTCCATGTGCCATGTAC AG-3' for reverse (R), 5'-AGTCGTCAGT(A/G)GTTCGAC-3' for F2 and 5'-CTCTGCAGCACCCTATCA-3' for R2 were used to test the other samples. Their equivalent positions in the NADL strain of BVDV-1 are 108–127 for F, 373–392 for R, 190–207 for F2 and 326–343 for R2, respectively. Primers were custom-made, and dissolved in sterile DEPC-treated water.

cDNA synthesis: The RT reaction solution was composed by 8 μl of 5X first strand buffer (250 mM Tris-HCl, pH 8.3, 375 mM KCl, 15 mM $MgCl_2$), 4 μl of 0.1 M dithiothreitol (DTT), 0.25 μl (25 U) of *Moloney murine leukemia virus* reverse transcriptase (Gibco BRL, Md., U.S.A.), 8 μl of deoxynucleotide triphosphates (dNTPs) to a final concentration of 0.2 mM each (Gibco-BRL), 0.2 μl (110 U/μl) of ribonuclease inhibitor, 0.25 μl (40 pM/μl) of primer R and DEPC-treated water to a final volume of 32 μl. Just before the incubation, 8 μl of RNA solution were added to 32 μl of RT solution and one drop of M-3516 mineral oil (Sigma Chemicals Co., Mo., U.S.A.) was added on the reaction mixture. The first strand synthesis of cDNA was performed in water bath at 37°C for 90 min.

PCR: The amplification of the 5'-UTR was performed according to the method described by Harasawa *et al.* [25]. Six microliters of cDNA solution were added to 5 μl of 10X buffer (100 mM Tris-HCl, pH 8.9, 800 mM KCl, 15 mM $MgCl_2$, 5 mg/m*l* bovine serum albumin, 1% sodium chloride, 1% Triton X-100), 1 μl (1.25 U) of *Thermus thermophilus* (*Tth*) DNA polymerase, 3 μl of *Tth* buffer (10 mM Tris HCl, pH 7.5, 300 mM KCl, 1 mM DTT, 0.1 mM disodium ethylenediaminetetracetate(EDTA), 50% glycerol), dNTPs to a final concentration of 0.2 mM each, 0.25 μl of F and R primers (40 pM/μl each) and water to a final volume of 50 μl. After the mixture was overlaid with mineral oil, the reaction cycle was carried out in a thermal cycler, 30 times with denaturation at 94°C for 30 sec, annealing at 55°C for 100 sec and extension at 72°C for 100 sec, 35 times with denaturation at 94°C for 30 sec, annealing at 52°C for 30 sec and extension at 72°C for 1 min, or 35 times with denaturation at 94°C for 1 min, annealing at 57°C for 1 min and extension at 72°C for 1 min. This amplified a product of about 285 base pairs from the cDNA. One microliter of the first step PCR product was used to perform the second step PCR with F2 and R2 primers and similar procedure. Tests were also performed using the method described by Vilcek *et al.* [45].

Agarose gel electrophoresis: Ten microliters of PCR products were mixed with 2 μl of 6X dye solution consisting of 0.25% xylene cyanol, 0.25% bromophenol blue and 40% sucrose in water. Molecular weight markers (MWM) from a *Hin*dIII- and *Hae*III- digested plasmid pHY containing nine fragments from 80 to 4,870 base-pairs or MWM XIV (Boehringer Manheim, Germany) were used to estimate the size of PCR products. One microliter of MWM was added to 2 μl of xylene cyanol-bromophenol blue dye solution and 9 μl of TE buffer (10 mM Tris-HCl,pH 8, 1 mM disodium EDTA). The 12 μl of each final sample was placed into the wells of the gel. One percent SeaKem medium electroendosmosis agarose gels (FMC Bioproducts, Me., U.S.A.), prepared with TAE buffer (40 mM Tris-HCl, pH 8.0, 5 mM sodium acetate, 1 mM disodium EDTA), were used to run an horizontal electrophoresis, submerged in TAE buffer, at 50 V constant for 45 min. The gels were stained in ethidium bromide solution (0.4 $\mu g/\mu l$) for 15 min in dark and destained 5 min in distilled water.

Nucleotide sequencing of PCR products: Agarose gel electrophoresis was performed with 10 m*l* of PCR products mixed with 2 μl of 6X dye solution. Molecular weight markers (MWM) containing 8 fragments from 65 to 2,364 base-pairs (Takara Shuzo, Japan) or 14 fragments (Boehringer Manheim, Germany) were used to estimate the size of PCR products. One microliter of MWM was added to 2 μl of xylene cyanol-bromophenol blue dye solution and 9 m*l* of TE buffer. The 12 μl of each sample solution were placed into the gel wells. Two percent NuSieve 3:1 low electroendosmosis agarose gel (FMC Bioproducts, Me., U.S.A.) in TAE buffer was used to run on horizontal electrophoresis, submerged in TAE buffer, at 100 V constant for 25 min. The gel was stained with ethidium bromide solution (2 $\mu g/\mu l$) for 10 min and destained 3 times with distilled water. The gel was observed using a 300 nm UV transilluminator. The portions of the gel containing positive bands were excised and DNA was extracted with Genetic II kit (BIO 100 Inc., U.S.A.). Three volumes of NAI solution were added and the gel was liquefied at 45°C incubation for 5 min. Glass-milk suspension was added and incubated for 5 min. After centrifugation for 5 sec, the supernatant fluid was discarded and the pellet was washed three times with fresh wash and eluted in 10 m*l* of distilled water. One microliter (equivalent to 3.2 pM) of primers R or F were mixed with 30–90 *n*g (determined by spectrophotometry) of purified DNA, 8 μl of pre-mix (Perkin-Elmer Corp., U.S.A.) consisting of 4 μl of 5X sequencing buffer, 1 μl of dNTP

mix, 0.5 μl of dye-deoxy terminators and 1 μl of Taq polymerase, and distilled water was added up to 20 μl of total volume. The mixture with each primer was vortexed for 1–2 sec, added with a drop of mineral oil and put in an ABI 9600 thermal cycler (Perkin-Elmer Corp., U.S.A.), with hot start at 95°C for 10 sec, then for 25 cycles of 96°C for 10 sec, 50°C for 5 sec and 60°C for 4 min. The amplified product (20 μl) was purified on Centri-Sep re-hydrated columns (Princeton Separations Inc., U.S.A.) with centrifugation at 3,000 rpm for 2 min in a VEC-100 vacuum evaporator centrifuge, dried for 15 min, denatured at 95°C for 2 min, chilled on ice for 10 min and transferred in an ABI sampling tube. Nucleotide sequencing of each PCR product was performed in an ABI Prism 310 Genetic Analyzer (Perkin-Elmer Corp., U.S.A.).

Comparison of primary and secondary structures of 5'-UTR: Primary structure in the 5'-UTR of the pestivirus strains detected in vaccine samples were compared with 18 published pestivirus sequences from North America, Europe and Japan, including international reference strains. Nucleotide sequences of the other pestivirus strains, obtained from the DNA databases, are as follows (accession number is given in parenthesis); strains NADL (M31182), SD-1 (M96751) and Oregon (L32876) for BVDV-1a, strains Osloss (M96687), Draper (L32880), Sanders (L20928) and NY-1 (L32879) for BVDV-1b, strains SE5572 (Z79770) and Europa (AB000898) for BVDV-1c, strains EBTr (D50817), 890 (L32886) and CD87 (L32887]) for BVDV-2, strains BD31 (U70263), Moredun cp (U65022) and Ch1Es (D50816) for BDV, and strains Brescia (AF091661), Alfort (J04358), and GPE- (AB019152) for CSFV. Nucleotide sequences of PCR products from the 5'-UTR of pestivirus RNA were aligned by the Clustal W [42]. A phylogenetic tree depending on the primary structures of the 5'-UTR among the known pestivirus RNA was constructed by the neighbor-joining method [39]. Secondary structures were predicted according to the algorithm of Zuker and Steigler [52]. Folding energies of the secondary structures were calculated by the method of Freier *et al.* [14]. Genotyping was performed according to the method described by Harasawa and Giangaspero [21]. Relevant variations in the characteristic three variable loci, V1, V2 and V3, conserved stem-loop palindromic sequences, at the 5'-UTR were considered for the phylogenetic analysis.

RESULTS

Five (13.1%) out of thirty-eight samples tested were positive for pestivirus or pestivirus RNA. Of the five positive specimens, three vaccines against measles (samples 1 and 12) and rubella (sample 5) were from Europe, and two vaccines against rubella (sample 8) and mumps (sample 10) were from Japan (Table 2).

Analyses based on primary nucleotide sequence homology and on secondary sequence structure of the 5'-untranslated genomic region of the contaminant pestivirus strains, revealed that the isolates belonged to the BVDV-1.

Comparison of the 5'-UTR nucleotide sequences from the five pestivirus contaminants with those from other 17 reference pestivirus sequences was performed by the Clustal W (Fig. 1). A phylogenetic tree based on primary nucleotide sequence comparison was obtained by the neighbor-joining method (Fig. 2).

Sequence variation in the 5'-UTR of the identified pestivirus strains, mainly limited to three variable loci V1, V2 and V3, were found by manual search, looking for palindromic nucleotide substitutions. Pestivirus characteristic consensus motifs at V1 and V2 levels (e.g. C:C base pairing and nucleotide sequence 5'-GGGGU-3' on the loop, respectively) were identified. The stable stem-loop structures showed substantial negative free energies: –51.06 KJ/mol V1, –51.06 KJ/mol V2 and –49.39 KJ/mol V3 in samples 1 and 5; –41.56 KJ/mol V1, –61.94 KJ/mol V2 and –21.34 KJ/mol V3 in sample 8; –37.25 KJ/mol V1, –84.13 KJ/mol V2 and –14.65 KJ/mol V3 in sample 10; –57.80 KJ/mol V2 and –29.74 KJ/mol V3 in sample 12.

Relevant variations of nucleotides were compared with typical nucleotides of the representative pestivirus genotypes. Nucleotide base pairs composing the palindromic stem structures, at the level of the three variable loci, V1, V2 and V3, at the 5'-UTR genomic region of the pestivirus strains isolated from a virus vaccine against measles (sample 1), and from a virus vaccine against rubella (sample 5) were identical and showed marked divergence from the previously described genotypes of pestiviruses (Fig. 3). The divergence was quantified and expressed in numbers of varying nucleotides (nt) or base pairs (bp) at the level of V1, V2 and V3 as follows: 8nt/5bp for BVDV-1a; 11 nt/7 bp for BVDV-1b; 10nt/7bp for BVDV-1c; 15nt/8bp for BVDV-2; 21nt/11bp for BDV; 23 nt/14 bp for CSFV. These specific aspects characterized a new genotype, BVDV-1d, presented in this study and described for the first time.

Tests revealed that BVDV-1d was genetically more related to BVDV-1a and BVDV-1b than the other pestiviruses. The nucleotide sequence values of the genotype BVDV-1d against genotypes BVDV-1a, BVDV-1b, BVDV-1c and BVDV-2 were of 82–86%, 83–86%, 81–83%, and of 71–74%, respectively. Similarly, the homologies between the BVDV-1d and BDV, and between the BVDV-1d and CSFV were less than 70%.

Palindromic structures at the 5'-UTR of the pestivirus strain isolated from a vaccine sample against measles (sample 12) showed clear similarities with BVDV-1b strains. The strain detected from sample 8 belonged to BVDV-1c and the strain from sample 10 to BVDV-1a (Fig. 3).

Comparison of the strains BRU*0615 (sample 8) and H503 (sample 10) with 64 reported-pestivirus sequences showed an overall nucleotide sequence homology with BVDV-1a, BVDV-1b, BVDV-1c and BVDV-2 were of 82–87%, 80–84%, 94–100% and 74–77%, and of 88–100%, 83–86%, 81–83% and 72%, respectively. With the strains from BDV and CSFV, homologies were less than 72%. The overall nucleotide sequence homology between the measles strain M98 (sample 12) and the BVDV-1a, BVDV-1b,

Table 2. Results of nested PCR tests for adventitious pestivirus RNA in live virus vaccines for human use

No	Virus antigen	1st PCR	2nd PCR	Genotype	Designation	Accession
1	measles	negative	positive	BVDV-1d	Massimo1	AB008838
2	measles	negative	negative			
3	MMR	negative	negative			
4	MMR	negative	negative			
5	rubella	negative	positive	BVDV-1d	Massimo2	AB008839
6	rubella	negative	negative			
7	measles	negative	negative			
8	rubella	negative	positive	BVDV-1c	BRU*0615	AB008837
9	rubella	negative	negative			
10	mumps	negative	positive	BVDV-1a	H503	AB008841
11	measles	negative	negative			
12	measles	negative	positive	BVDV-1b	M98	AB014339
13	measles	negative	negative			
14	measles	negative	negative			
15	measles	negative	negative			
16	MMR	negative	negative			
17	MMR	negative	negative			
18	MMR	negative	negative			
19	MMR	negative	negative			
20	MMR	negative	negative			
21	MMR	negative	negative			
22	rubella	negative	negative			
23	rubella	negative	negative			
24	rubella	negative	negative			
25	rubella	negative	negative			
26	rubella	negative	negative			
27	polio	negative	negative			
28	polio	negative	negative			
29	polio	negative	negative			
30	polio	negative	negative			
31	polio	negative	negative			
32	polio	negative	negative			
33	polio	negative	negative			
34	polio	negative	negative			
35	polio	negative	negative			
36	polio	negative	negative			
37	polio	negative	negative			
38	Str. pneumoniae*	negative	negative			

*: Bacterial vaccine.

BVDV-1c and BVDV-2 were 73–85%, 95–97%, 87–89% and 63–64%, respectively.

The nucleotide sequence of the genomic 5'-UTR of the pestivirus strains, presented first in this study, have been deposited in the DDBJ, EMBL and GenBank nucleotide sequence databases under the accession numbers AB008837 (BRU*0615), AB008838 (Massimo1), AB008839 (Massimo2), AB008841 (H503) and AB014339 (M98).

DISCUSSION

The results obtained during this study provided evidence for the occurrence of pestivirus or pestivirus RNA contamination in live virus vaccines for human use from Europe and Japan.

Analyses based on primary nucleotide sequence similarity and on secondary structure in the 5'-UTR revealed that all the contaminants detected by RT PCR belonged to BVDV-1. Three RNA sequences were classified into the BVDV-1a, BVDV-1b and BVDV-1c genotypes. Two RNA sequences, detected from measles and rubella vaccine samples from Europe, were characteristic for a new genotype of BVDV-1: genotype BVDV-1d. The results obtained by the evaluation of the sequence divergence at secondary structure level, at the 3 variable loci, palindromic structures, in the 5'-UTR were comparable to those obtained by the nucleotide sequence alignment and the phylogenetic tree obtained from comparison of the nucleotide sequences in the 5'-UTR among known pestivirus RNA.

The 5'-UTR of the pestivirus includes regulatory motifs necessary for viral gene expression and RNA replication, which are usually composed of a combination of primary, secondary and tertiary structures [12, 27]. The nucleotide sequence of the 5'-UTR is very similar and well conserved among the members of the *Pestivirus* genus [29, 35], thus providing a useful region suitable for accurate virological

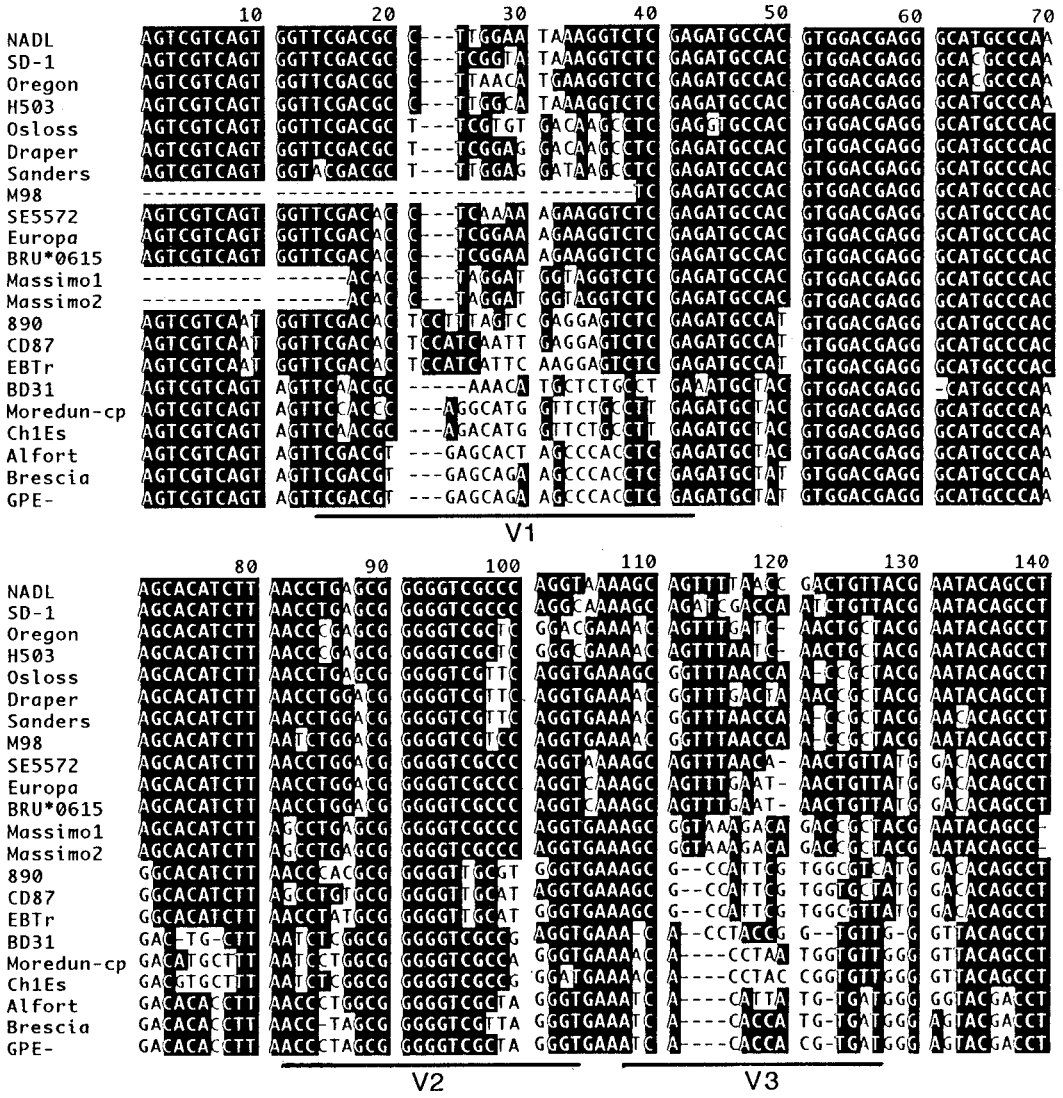

Fig. 1. Nucleotide sequence alignment of the PCR products from the 5'-UTR for sequence comparison of the virus vaccine contaminant strains with 17 reference pestivirus sequences. Nucleotide sequences of BVDV, BDV and CSFV strains are used for references. Nucleotides that are identical in two out of three sequences are shown in white characters. The nucleotide sequence numbers are given from a consensus alignment. Dashes represent spacers between adjacent nucleotides introduced for maximum alignment.

diagnosis and other peculiar studies, including the taxonomy of pestivirus.

In the present study, analysis of the 5'-UTR based on the primary structure were used for species identification of pestivirus. Furthermore, we additionally applied the palindromic nucleotide substitution (PNS) genotyping method, a new procedure based on the predicted secondary structures in the 5'-UTR, simple and meaningful approach, providing results corresponding to those obtained by the other methods, through specific evaluation of nucleotide sequence variations at the level of three characteristic palindromic structures [21].

The PNS method, through determining genetic variation among strains, can be used as genetic marker to determine the appurtenance to the genus *Pestivirus*, the species and the genotype. The observation obtained through PNS indicates that BVDV-1a, BVDV-1b, BVDV-1c, and BVDV-1d are genotypes of the BVDV-1 species. However, it is still necessary to clarify a better definition of pestivirus species and genotypes. In addition, among strains belonging to BVDV-1 there exists an important variability.

Tests were done in Belgium, Germany, Italy and Japan by five experienced institutions and national reference centers for pestivirus detection. A double check on the same sam-

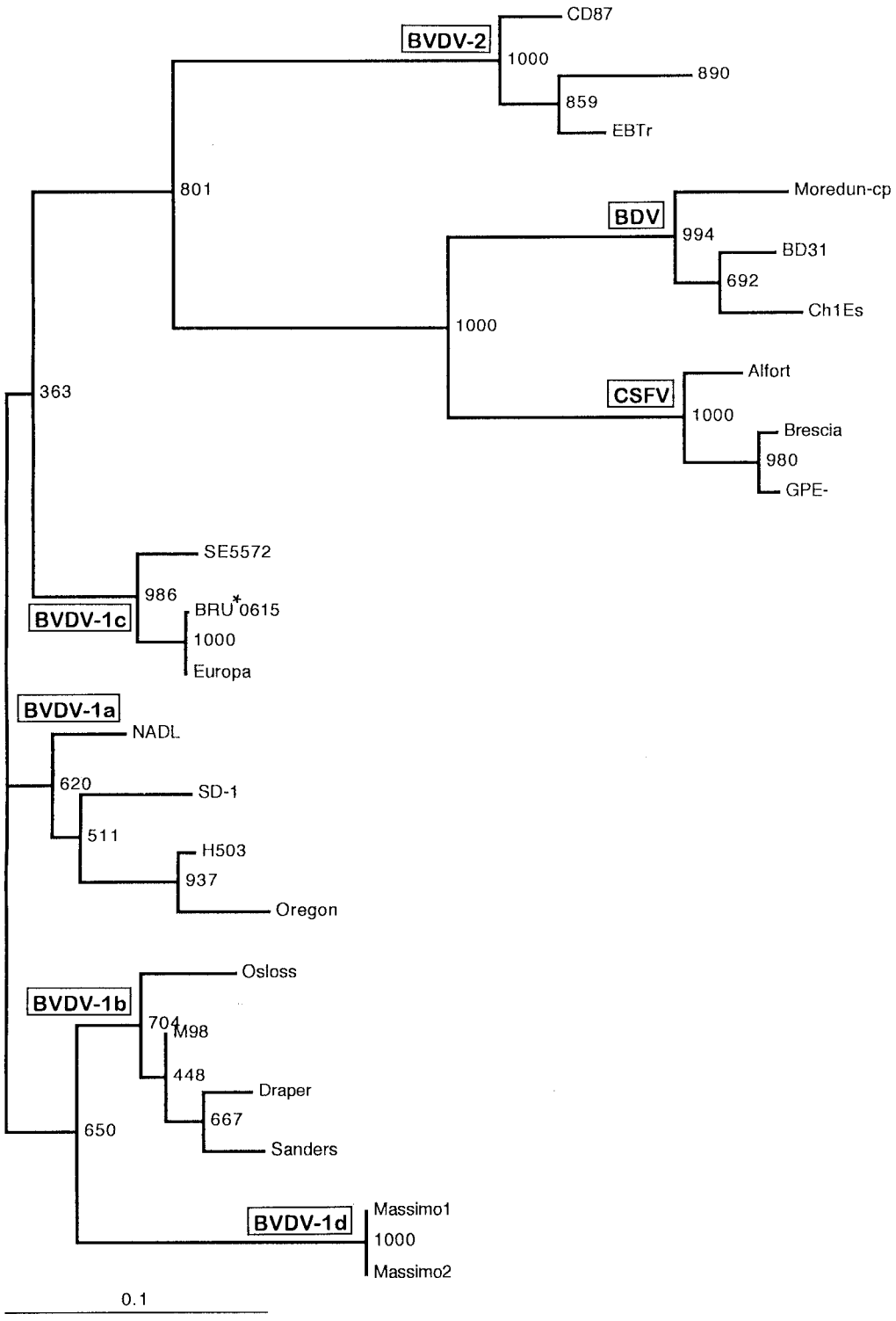

Fig. 2. Phylogenetic tree based on 5'-UTR sequence comparison from different pestivirus strains, obtained by the neighbor-joining method. Numbers at the relevant branches refer to bootstrap values of 1,000 replications. Scale bar indicates 10-nucleotide substitutions per 100 nucleotides.

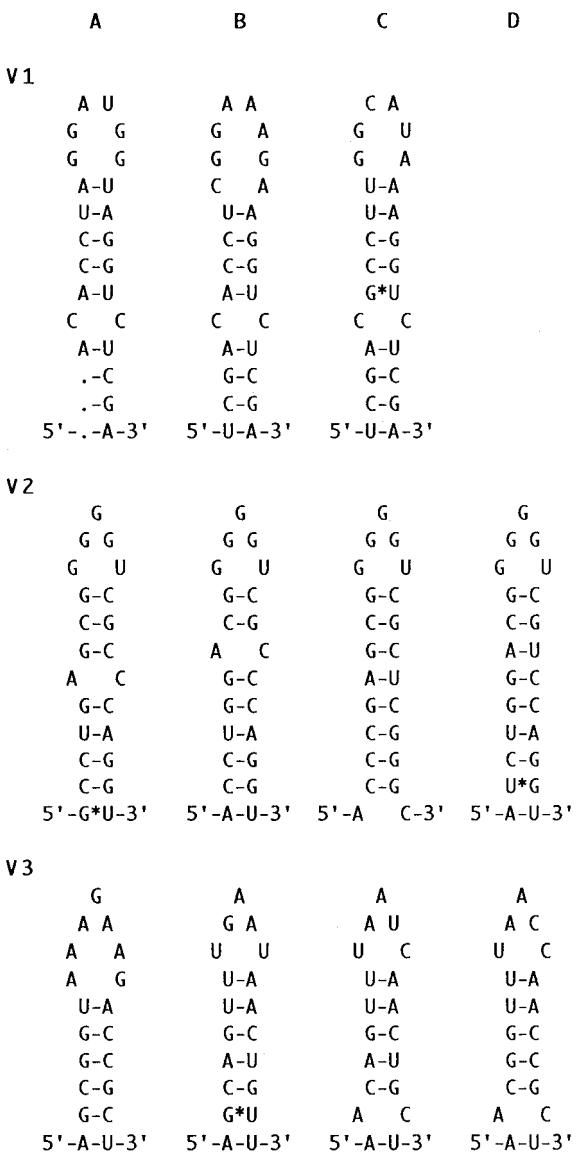

Fig. 3. Palindromic structures (V1, V2 and V3) in the 5'-UTR genomic region of the pestivirus strains isolated from virus vaccines. Lane A represents secondary structures of contaminants from measles (sample 1) and rubella (sample 5) vaccines, lane B represents those from a rubella vaccine (sample 8) , lane C represents those from a mumps vaccine (sample 10) , lane D represents those from a measles vaccine (sample 12). Watson-Crick base pairing is shown by a dash (-), and G:U pairing tolerated in secondary structures is shown by an asterisk (*).

ples was performed in two different laboratories. Results obtained on samples 15, 20, 26, 32 and 37, all negative, when tested by one of the five laboratories, were confirmed by re-testing the extracted RNA by another laboratory.

However, PCR tests performed on the vaccine samples belonging to the same lots did not show corresponding results. Giving the supposed low titres of virus contaminants, as suggested by the negative results obtained with the first-step PCR products, this discordance was probably due to the heterogeneity of distribution of the contaminant pestivirus RNA in the different vials originating from the same production lot. Consequently, unless a sample pooling followed by concentration is made, single vials may contain virus concentrations lower then the sensitivity threshold of the PCR diagnostic system. This implies that, despite belonging to a same lot, each vaccine vial should be considered as a different sample.

In three experiments, we used a positive control, the BVDV reference strains Oregon, NY-1 and NADL. This raised the necessity to increase accuracy of the test to avoid any risk of contamination. In the test with NY-1 strain of BVDV-1b, the characterization of a positive sample (number 12) showed its appurtenance to the same BVDV-1b genotype, but nucleotide sequence divergence was evident and excluded the occurrence of cross contamination. The test with NADL strain did not reveal positive samples. However, in further analyses, it will be easier to use an internal positive control which contains the primer binding sites but will yield a larger product to clearly discriminate from pestivirus specific amplifications.

The percentage of positive samples reported by Harasawa and Tomiyama [24] in 1994, 4 (80%) positive out of 5 tested, was not representative because of the reduced number of samples evaluated. In the second experiment, the positive rate was 12 (27.9%) out of 43 lots of human live virus vaccines produced by six Japanese manufactures [40]. An examination on interferon for human use showed a percentage of 30.4% out of 46 tested samples [23]. The positiveness revealed in the current study was lower. The negative results recently reported by Vilcek *et al.* [46] on European human virus vaccines did not allow to further conclusion. Only through a wider screening it will be possible to obtain a clear evaluation of the degree of the problem.

A topic of interest is the identification of sources of viral contaminants. During the previous control experiments undertaken in Japan [24], FBS was used as medium supplement for the cell cultures used for the production of the vaccines for human use which were contaminated by pestivirus or pestivirus RNA and it was the most probable source of contamination. This circumstance could be occurred also in the actual case with vaccines produced in cell substrates supplemented with contaminated FBS.

Notwithstanding the high production and control standards applied in Japan and Europe, there arises the problem about safety of the actual production methods of biological products. Thermal inactivation associated with β-propiolactone treatment was proposed to inactivate BVDV contaminating FBS [6]. Bolin *et al.* [3] reported BVDV contamination in commercialized FBS treated with the above mentioned method. Furthermore, standard procedures (e.g. indirect immune fluorescence and serum neutralization tests), when applied without prior concentration of high volumes, especially when low virus titres are involved, might be inefficient. Bolin *et al.* [5] reported that BVDV

RNA detection by PCR from experimentally infected monkey cells was unsuccessful unless sequential passages on turbinate bovine cells were made.

The detection of pestivirus RNA in live virus vaccines for human use does not necessarily mean the presence of infectious virions, but occurrence of iatrogenic infection in animals has been reported, in relation with vaccines for veterinary use contaminated by infectious pestivirus [13, 26, 28, 43, 47].

The role of pestiviruses in human pathology remains unknown. So far, no direct relationship between pestivirus infections in animals and human clinical diseases, especially in risk categories (e.g. farm workers, animal caretakers, veterinarians), has been proved. However, possible indirect impacts on human health through immunodepression or forms of synergism with other pathogens cannot be excluded. These aspects are difficult to identify and to investigate as epidemiological pictures far from any clearness. Nevertheless, antibodies against BVDV were detected in humans from Europe [15, 17, 18], U.S.A. [34, 50, 51], Bangladesh [50] and New Zealand [48]. In addition, there exist indications suggesting that pestivirus can be a risk for emerging infections in humans through congenital and acquired nervous pathologies [15, 34, 51], gastrointestinal and respiratory disorders [50] and eventual relationship with HIV infection [17]. However, at present, the pestivirus infection cannot be associated to human illness. The danger that BVDV represents for cattle through infections characterized by an important immunodepressive action has to be taken into account.

In conclusion, these preliminary results confirmed the previous observations obtained in Japan and indicated that the occurrence of pestivirus contamination in biological products for human use seems to be an actual problem with unknown effect on human health.

The importance of these findings rely on the evident contrast with the general rules on the safety of pharmaceutical products for human use, which clearly excludes any kind of contamination. Therefore, monitoring for pestivirus contamination should be recommended on biologicals for human use.

Finally a contribution to a better definition of BVDV-1 genotype was given by the characterization for a new genotype BVDV-1d, presented in this study, and described for the first time through the PNS genotyping method, based on the analysis of palindromic secondary structures of the 5'-UTR.

The evaluation of the presence of live infectious pestivirus contaminating biological products for human use will be subject of future studies.

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