Biologicals xxx (xxxx) xxx–xxx

Contents lists available at ScienceDirect

Biologicals

journal homepage: www.elsevier.com/locate/biologicals

Detection of bovine viral diarrhoea virus nucleic acid, but not infectious virus, in bovine serum used for human vaccine manufacture

Majid Laassri[a], Edward T. Mee[b], Sarah M. Connaughton[b], Hasmik Manukyan[a], Marion Gruber[a], Carmen Rodriguez-Hernandez[c], Philip D. Minor[b], Silke Schepelmann[b], Konstantin Chumakov[a,*], David J. Wood[c]

[a] FDA Center for Biologics Evaluation and Research, 10903 New Hampshire Avenue, Silver Spring, MD, 20993, United Kingdom
[b] Division of Virology, National Institute for Biological Standards and Control, Medicines and Healthcare Products Regulatory Agency, South Mimms, EN6 3QG, United Kingdom
[c] Department of Essential Medicines and Health Products, World Health Organization, Avenue Appia 22, Geneva, Switzerland

ARTICLE INFO

Keywords:
Adventitious agents
PCR
Deep sequencing
Bovine serum
Immunofluorescence
Virus replication assay

ABSTRACT

Bovine viral diarrhoea virus (BVDV) is a cattle pathogen that has previously been reported to be present in bovine raw materials used in the manufacture of biological products for human use. Seven lots of trivalent measles, mumps and rubella (MMR) vaccine and 1 lot of measles vaccine from the same manufacturer, together with 17 lots of foetal bovine serum (FBS) from different vendors, 4 lots of horse serum, 2 lots of bovine trypsin and 5 lots of porcine trypsin were analysed for BVDV using recently developed techniques, including PCR assays for BVDV detection, a qRT-PCR and immunofluorescence-based virus replication assays, and deep sequencing to identify and genotype BVDV genomes.

All FBS lots and one lot of bovine-derived trypsin were PCR-positive for the presence of BVDV genome; in contrast all vaccine lots and the other samples were negative. qRT-PCR based virus replication assay and immunofluorescence-based infection assay detected no infectious BVDV in the PCR-positive samples. Complete BVDV genomes were generated from FBS samples by deep sequencing, and all were BVDV type 1. These data confirmed that BVDV nucleic acid may be present in bovine-derived raw materials, but no infectious virus or genomic RNA was detected in the final vaccine products.

1. Introduction

The use of animal- or human-derived cell lines and reagents for the production of vaccines and biotherapeutics carries the risk of propagation of adventitious viruses during cell culture production stages and contamination of the final products. Stringent adventitious virus testing is carried out on raw materials, cell banks and vaccine seed stocks [1] however such tests may fail to detect viruses without the relevant host tropism during testing, or those that do not display a cytopathic effect *in vitro* or morbidity/mortality *in vivo*. Modern molecular methods such as microarray hybridization and metagenomics offer a broader range of detection and the possibility to detect contaminants missed by conventional screening. For example, Porcine Circovirus DNA was detected by deep sequencing in two licensed live viral vaccines – and infectious virus in one – despite the relevant safety tests having returned satisfactory results [2–4].

The use of bovine serum as a supplement in cell culture is one of the potential sources for adventitious virus contamination. The use of foetal – rather than new-born or adult - serum reduces the risk of contamination; however a number of viruses may still be present. A variety of virus reduction/removal methods, e.g. heat inactivation or filtration may be employed to reduce the risk of the virus amplifying and persisting through to the final production stages, however nucleic acid and potentially infectious virus may persist depending on the initial virus burden and the stringency of the methods used.

One of the more common contaminants of bovine serum is Bovine Viral Diarrhoea Virus (BVDV), which causes endemic infection of cattle in many countries [reviewed in Ref. [5]]. BVDV is a pestivirus of the family *Flaviviridae* [6]. It is an enveloped, positive sense single-stranded RNA virus with a size of 40–60 nm. More than 80% of cattle are seropositive against the virus. BVDV is genetically similar to HoBi-like viruses [7] and can be divided into two types based on its effect on cell cultures; cytopathic (CP) and noncytopathic (NCP), and into three genotypes (1, 2 and 3), based on genetic variations and antigenic

* Corresponding author.
E-mail address: konstantin.chumakov@fda.hhs.gov (K. Chumakov).

https://doi.org/10.1016/j.biologicals.2018.06.002
Received 7 August 2017; Received in revised form 13 June 2018; Accepted 15 June 2018

distribution [reviewed in Refs. [8,9]].

NCP viruses are exclusively type 1; they cause little disease in cattle, and infections during pregnancy may lead to persistent infection (PI) in newborn calves [10,11]. The infected foetuses are the source of FBS contamination [10], and because NCP viruses are responsible for more than 80% of natural BVDV infections, they are the principal BVDV type present in FBS. A survey of cell lines in ATCC showed NCP viruses as a major contaminants of cell lines derived from various animal species, whilst human cell lines were found to be non-permissive [12].

Previous studies showed some human live viral vaccines were contaminated by BVDV-1 RNA coming from bovine-derived raw materials used in the manufacture [13–16]. The vaccines in these studies were monovalent mumps, monovalent rubella and two combined measles, mumps and rubella vaccines produced by different manufactures [13,14]. In another study, interferon produced by different pharmaceutical companies for human use was found to be contaminated by BVDV RNA, with 86% being BVDV-1 [17]. In general measles, mumps and rubella vaccines and combined (MMR) vaccines are manufactured under requirements of good manufacturing practices for pharmaceutical and biological products. Sera used for the propagation of cells for these vaccines production are treated to inactivate live virus and tested to demonstrate freedom from viruses, bacteria, fungi and mycoplasmas as specified in general requirements for the sterility of biological substances [18].

BVDV is not known to cause disease in humans. BVDV antibodies have previously been detected in samples collected from human patients, although causative links between exposure to BVDV and diseases have not been established; two serum samples collected from mothers with microcephalic infants were positive for BVDV-1 (NADL strain) antibodies [19]. Similarly, BVDV antibodies were found in 40% of sera collected from twins incongruous for schizophrenia [20]. Another study showed the presence of BVDV antigen in 23.6% of faecal samples collected from children suffering from gastroenteritis [21]. Recently, unpublished data from proteomics analysis of brain tissue samples from foetuses bearing microcephaly during the 2015 Zika outbreak in Brazil was reported to suggest the presence of peptide(s) from the polyprotein of a BVDV-like virus, although they were also related to peptides from host ubiquitin [22,23]. This led to speculation that Zika virus may act together with BVDV infection to cause microcephaly in human foetuses [24]. There is no evidence of BVDV playing a role in microcephaly [25], however, it is prudent to explore sources of potential BVDV infection, including vaccines.

We evaluated the presence and infectious status of BVDV in monovalent and trivalent MMR vaccines, because live viral vaccines are more vulnerable to contamination with adventitious agents than inactivated vaccines [2]. We also studied a range of bovine sera, horse serum, bovine and porcine trypsin that are used in manufacture of vaccines and could be a source of contamination with adventitious agents. Viral RNA was detected in all bovine serum lots and one of two bovine trypsin lots, but not in horse serum, porcine trypsin or any vaccine lots. Infectious virus was not detected in any of the samples tested. Our results confirm previous findings of a high prevalence of BVDV nucleic acid in bovine serum [26,27] but showed no evidence of infectious virus in serum used for vaccine manufacture. Further, viral RNA did not persist through the manufacturing process to the final vaccine product.

2. Materials and methods

2.1. Viruses, FBS, vaccines and cells

VDV isolate Ky1203 (noncytopathic) and BVDV-free Madin Darby Bovine Kidney (MDBK) cells were a kind gift from Dr. Carole Thomas (Royal Veterinary College, UK). The Bovine Viral Diarrhea virus (BVDV) reference strain was kindly provided by Dr. Judy Beeler (CBER/FDA). BVDV-infected RK-13 cells were available at NIBSC. Vaccine and serum included one batch of measles vaccine and 7 batches of MMR vaccines from the same manufacturer, and two lots of serum from the vaccine manufacturing process - FBS M1 and M2. Serum, vaccines and reagents were purchased from different vendors (Table 2). MDBK cells were maintained in Minimal Essential Medium (Sigma-Aldrich) containing 2% GlutaMAX (Life Technologies), 2% foetal calf serum, 1% penicillin/streptomycin (Sigma-Aldrich) and 1% amphotericin B (Sigma-Aldrich).

2.2. Sample preparation

Three batches of MMR single dose vials and four batches of ten-dose vials were reconstituted in 500 µl manufacturer-supplied water for injection. Serum samples and culture supernatants clarified by centrifugation at 400 × g for 5 min were processed neat. Cell pellets were prepared by rinsing of confluent flasks with serum-free culture media, scraping cells into phosphate buffered saline and clarifying at 400 × g for 5 min.

2.3. RNA extraction

A 140 µl volume of each vaccine, serum sample or culture supernatant was processed using the QIAamp vRNA Mini Spin Kit (Qiagen) according to the manufacturer's method and with the inclusion of carrier RNA. RNA was eluted in 60 µl buffer AVE. Parallel extraction controls included water for injection and RT-PCR grade water (Ambion).

Cell pellets were resuspended in 200 µl phosphate buffered saline and RNA was extracted using the High Pure RNA Isolation kit (Roche). RNA was eluted in 100 µl of the supplied elution buffer. Extracted RNA samples were stored at −20 °C prior to PCR.

2.4. Reverse transcriptase polymerase chain reaction (RT-PCR)

Primers and expected DNA amplicon sizes are described in Table 1. Amplification was performed using SuperScript III OneStep RT-PCR kit with Platinum Taq (Invitrogen), using 0.4 µM each primer, 5 µl of eluted RNA, 1× reaction mix and 0.5 µl of enzyme mix in a total volume of 25 µl. Reverse transcription was performed at 50 °C for 30 min followed by denaturation at 94 °C for 2 min. PCR was performed using 40 cycles of: 94 °C for 15 s, 60 °C for 30 s, 68 °C for 30 s and a final extension at 68 °C for 5 min. Amplicons were visualised by mixing 5 µl of each reaction with 1 µ l of 6 × DNA Gel Loading Dye (ThermoFisher), separating at 100 V for 30–60 min on a 2% agarose (Bioline) gel containing 1× SybrSafe DNA Gel Stain (ThermoFisher) and imaging by ultraviolet transillumination.

2.5. Nested PCR

Total RNA was isolated as described above. The nested PCR primers are listed in Table 1. F1 and R1 were used in the first-step PCR reaction, and primers F2 and R2 were used in the second-step PCR reaction. These primers were described previously [28] and were designed from genomic sequences of high homology among BVDV strains. The first-step PCR amplification was carried out with OneStep RT-PCR kit (Qiagen) in 50 µl reaction mixtures containing 3 µl of RNA solution, 10 µl of 5× OneStep RT-PCR Buffer, 2 µl OneStep RT-PCR Enzyme Mix, dNTPs to a final concentration of 0.4 mM each, 0.6 µM as final concentration of each F1 and R1 primers, and water to a final volume of 50 µl. Amplification was performed in a Veriti 96 well thermal cycler (Applied Biosystems). Thermal cycling parameters were as follows: reverse transcription at 50 °C for 30 min, PCR activation at 95 °C for 15 min, followed by 40 cycles of denaturation at 95 °C for 30 s, annealing at 56 °C for 30 s, and extension at 72 °C for 30 s.

The first-step PCR products were diluted 1:1000 with sterile distilled water and were subjected to the second-step PCR using HotStar