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Metagenomic assessment of adventitious viruses in commercial bovine sera

Kathy Toohey-Kurth [a, b], Samuel D. Sibley [a], Tony L. Goldberg [a, c, *]

[a] University of Wisconsin-Madison, Department of Pathobiological Sciences, 1656 Linden Drive, Madison, WI 53706, USA
[b] Wisconsin Veterinary Diagnostic Laboratory, 445 Easterday Lane, Madison, WI 53706, USA
[c] University of Wisconsin-Madison Global Health Institute, 1300 University Avenue, Madison, WI 53706, USA

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ABSTRACT

Animal serum is an essential supplement for cell culture media. Contamination of animal serum with adventitious viruses has led to major regulatory action and product recalls. We used metagenomic methods to detect and characterize viral contaminants in 26 bovine serum samples from 12 manufacturers. Across samples, we detected sequences with homology to 20 viruses at depths of up to 50,000 viral reads per million. The viruses detected represented nine viral families plus four taxonomically unassigned viruses and had both RNA genomes and DNA genomes. Sequences ranged from 28% to 96% similar at the amino acid level to viruses in the GenBank database. The number of viruses varied from zero to 11 among samples and from one to 11 among suppliers, with only one product from one supplier being entirely "clean." For one common adventitious virus, bovine viral diarrhea virus (BVDV), abundance estimates calculated from metagenomic data (viral reads per million) closely corresponded to Ct values from quantitative real-time reverse transcription polymerase chain reaction (rtq-PCR), with metagenomics being approximately as sensitive as rtq-PCR. Metagenomics is useful for detecting taxonomically and genetically diverse adventitious viruses in commercial serum products, and it provides sensitive and quantitative information.
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1. Introduction

Animal serum is an essential supplement for cell culture media, making possible applications such as virus isolation (e.g. for diagnostics) and virus propagation (e.g. for vaccine production). It is also used in animal breeding as a component of media for gamete and embryo storage during artificial insemination and embryo transfer, respectively [1]. Although fetal bovine serum (FBS) is the most widely used such product, other products include serum collected from newborn calves and from "donor" cows raised in bio-secure facilities [2]. Serum from other species (e.g. goats, horses) is a viable alternative for certain applications [3,4], but bovine serum remains the industry standard. Considerable effort has been devoted to developing artificial replacements for animal serum, but this task has proven difficult due to the many biologically active molecules in serum that are necessary for the growth and survival of cells *in vitro* [5–7].

Among microbial contaminants of commercial animal serum, viruses are especially problematic because they are difficult to detect and they are not removed by sterile filtration [8–10]. Consequently, panels of tests must be conducted on commercial sera to ensure quality and to meet regulatory requirements [11]. Even with such testing, viral contamination of commercial serum still occurs. For example, bovine viral diarrhea virus (BVDV; *Flaviviridae*, *Pestivirus*) is a pervasive contaminant of animal and human vaccines, having led in some cases to economically costly product recalls [12–14]. Gamma and ultraviolet radiation can inactivate viruses in sera without destroying the biologically active molecules that potentiate cell survival and growth, but their efficiency varies with virion properties and specialized equipment and facilities may be required [15,16]. As a result, the primary assurances against viral contamination of serum products remain good manufacturing practices and extensive testing for specific viral agents [17]. Other safeguards, such as selective geographic sourcing, appear to be less reliable [18].

Metagenomics, made possible by next generation DNA sequencing, has led to a revolution in virus detection [19]. Unlike

* Corresponding author. University of Wisconsin-Madison, Department of Pathobiological Sciences, 1656 Linden Drive, Madison, WI 53706, USA.
E-mail address: tony.goldberg@wisc.edu (T.L. Goldberg).

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polymerase chain reaction (PCR) or other methods that rely on the presence of known sequences, metagenomic viral detection is "unbiased," in that it does not require *a priori* knowledge of which viruses may be present or their nucleic acid sequences [20]. Instead, these methods rely on a random sequencing approach in which libraries of DNA (or reverse-transcribed RNA) are subjected to massively parallel DNA sequencing, with bioinformatics analyses subsequently applied to detect sequences matching those in databases of known viruses [21]. We have employed such methods successfully to detect a variety of pathogens (viral and otherwise) in the sera and other tissues of animals [e.g. Refs. [22–26].

Here, we describe the application of metagenomic methods for the detection of viruses in bovine sera obtained from commercial sources. Our analysis expands on previous work suggesting that such approaches might be useful for detecting adventitious viruses in biologics such as sera and trypsin [20,27,28]. Our results have implications for metagenomics as a quantitative tool, as well as a qualitative one, and for quality control during the manufacture of serum and serum-derived biological products.

2. Materials and methods

2.1. Sources of sera

We obtained 26 commercial sera from 12 independent manufacturers in the USA, Australia and New Zealand, including 20 FBS, five donor cow sera, and one newborn calf serum. After receipt by courier, aliquots of 1.0 ml of each product were placed in nuclease-free microcentrifuge tubes and stored at −80 °C prior to metagenomic analysis.

2.2. Virus detection

Each 1.0 ml serum aliquot was clarified and treated with nucleases [29], after which viral RNA was isolated using the Qiagen QIAamp MinElute virus spin kit (Qiagen, Hilden, Germany), omitting carrier RNA. Extracted nucleic acids were then converted to double-stranded cDNA using the Superscript double-stranded cDNA Synthesis kit (Invitrogen, Carlsbad, CA, USA) and random hexamers. Resulting cDNA was purified using Agencourt Ampure XP beads (Beckman Coulter, Brea, CA, USA), and approximately 1 ng DNA was prepared as a library for pair-ended sequencing on an Illumina MiSeq instrument (MiSeq Reagent kit v3, 150 cycle, Illumina, San Diego, CA, USA) using the Nextera XT DNA sample preparation kit (Illumina, San Diego, CA, USA). Real-time quantitative reverse transcription PCR (rt-qPCR) data on BVDV (types 1 and 2) were conducted by the Wisconsin Veterinary Diagnostic Laboratory using published methods [30].

2.3. Bioinformatics

Data were analyzed for viral sequences using CLC Genomics Workbench version 8.5 (CLC bio, Aarhus, Denmark). Briefly, low-quality bases were trimmed (phred quality score < 30) and short reads (<25 bp) were discarded, sequences of known contaminants of molecular biology reagents were removed, and remaining reads were subjected to *de novo* assembly. Raw reads and assembled contiguous sequences (contigs) were analyzed for similarity to viruses (excluding bacteriophages) in the GenBank database at the nucleotide (BLASTn) and amino acid (BLASTx) levels [31,32]. Contigs with homology to viruses in GenBank were extracted and compared to the most similar published virus genome using similarity statistics and length of sequence coverage as metrics. All reads were also aligned with ("mapped" to) viral sequences downloaded from Genbank (last accessed August 2016) using strict mapping parameters (70% of the read length, 80% identity to the reference sequence). Based upon the resulting information, a database of virus sequences present was compiled. All reads and contigs for each sample were then mapped to this database. Consensus sequences from each sample were then extracted and compared to reference sequences using BLASTx to determine percent ID and hit lengths. Finally, the abundance of each viral genome in each sample was measured as proportion of reads mapping to the consensus sequence of that virus, normalized to 1 million reads.

2.4. Data availability

All sequence data and metadata are available in the NCBI BioSample database (http://www.ncbi.nlm.nih.gov/biosample/) under BioProject PRJNA343833, BioSample accession numbers SAMN05804635 – SAMN05804660.

3. Results

3.1. Detection of viruses

We detected viral sequences in all but one of the 26 commercial serum samples. Viruses detected were members of 10 families, with genomes of single stranded DNA, double stranded DNA, single stranded RNA, and double stranded RNA (Table 1). Assembly of distinct, biologically relevant (*i.e.,* non-chimeric) viral genomes was not possible because of the large number of variably related viral sequences in each serum sample (commercial sera are pools of sera from many different animals). Therefore, similarity of viruses in each sample to viruses in the GenBank database was based on the consensus sequence derived from both raw reads and contiguous sequences (contigs), extracted from each mapping file as described above. Resulting sequence similarities to known viruses ranged from 28% to 98% of amino acids (Table 1). Bovine viral diarrhea virus (BVDV, types 1 and 2; *Flaviviridae*, *Pestivirus*) was the most common virus detected, followed by various parvoviruses (*Parvoviridae; Parvovirus*) (Table 1).

3.2. Viral abundance

Raw numbers of sequences acquired per sample ranged between 948141 and 2143615, with viral reads ranging from zero to approximately 50,000 per million per sample (Fig. 1). The most abundant viruses were BVDV and the parvoviruses, which were also the most commonly detected viruses, as described above. All samples identified as negative by rt-qPCR had BVDV read counts ranging from zero to 36 per 10^6, consistent with the well known phenomenon of "read bleed," in which small numbers of sequences are mis-assigned due to particularities of the Illumina sequencing technology [33]. Samples that were positive for BVDV by rt-qPCR had read counts ranging from 42 to 38,118 per 10^6. For BVDV-positive samples, the correlation between Ct values and read counts (normalized to 10^6) showed a strong and statistically significant log-linear relationship ($r = -0.82$), with the limit of detection by rt-qPCR (a Ct value of approximately 35) corresponding to approximately 10^2 reads per million (Fig. 2).

3.3. Variation among suppliers

Viral contamination varied markedly among suppliers. Certain suppliers (e.g. G and J in Fig. 1) had relatively low levels of contamination, whereas others (e.g. A, B, I and L in Fig. 1) had high viral diversities and abundances. Only one product from one supplier was entirely "clean" (Sample 23 from supplier J in Fig. 1). Viral

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