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Metagenomic assessment of adventitious viruses in commercial bovine sera

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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.

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

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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

Table 1
Adventitious viruses detected in 26 samples of commercial bovine serum using metagenomics.

	Virus	Family	Genome	Accession[a]	Samples[b]	Hits[c]	Hit length[d]	% identity[e]
1	Chimpanzee faeces associated circular DNA molecule 1	unassigned	ssDNA	YP_009259695	1	1	277	28.0
2	Rodent stool-associated circular genome virus		ssDNA	AEM05798	1	1	300	30.8
3	Bovine faeces associated circular DNA virus 1		ssDNA	YP_009252327	1	1	282	37.7
4	Lake Sarah-associated circular virus-27		ssDNA	YP_009237592	1	1	258	40.1
5	Gorilla smacovirus		ssDNA	AJF23087	1	2	172 (21–323)	69.5 (53.2–85.7)
6	Norovirus genogroup 3	*Caliciviridae*	ssRNA	AY274819	2	30	85.8 (15–709)	96.4 (83.8–100)
7	Empeyrat virus	*Dicistroviridae*	ssRNA	AMO03208	1	1	845	32.7
8	Bovine viral diarrhea virus 1	*Flaviviridae*	ssRNA	NC_001461	17	639	149.0 (13–3889)	94.2 (70.0–100)
9	Bovine viral diarrhea virus 2		ssRNA	NC_002032	3	177	100.4 (15–3896)	94.9 (66.7–100)
10	Porcine parvovirus 6	*Parvoviridae*	ssDNA	ALJ96701	2	4	251.8 (50–504)	45.6 (39.6–58.5)
11	Porcine parvovirus 4		ssDNA	AGM20656	5	18	120.2 (23–401)	58.3 (31.5–100)
12	Adeno-associated virus		ssDNA	NC_005889	1	17	73.9 (19–473)	87.4 (58.3–100)
13	Bovine parvovirus 2		ssDNA	YP_077176	11	137	119.7 (16–911)	90.3 (31.3–100)
14	Bovine hokovirus 2 strain HK-B38		ssDNA	JF504698	1	7	71.0 (16–583)	95.6 (32.4–100)
15	Bovine parvovirus 3		ssDNA	AF406967	2	97	53.5 (17–948)	97.6 (56.0–100)
16	Human picobirnavirus	*Picobirnaviridae*	dsRNA	AB517739	1	12	42.5 (17–213)	85.2 (61.9–100)
17	Bovine kobuvirus	*Picornaviridae*	ssRNA	NC_004421	1	1	2464	96.4
18	Bovine polyomavirus	*Polyomaviridae*	dsDNA	NC_001442	2	150	47.3 (12–357)	98.6 (38.0–100)
19	Epizootic hemorrhagic disease virus	*Reoviridae*	dsRNA	NC_013396	1	27	38.2 (19–114)	91.1 (35.4–100)
20	Bovine leukemia virus	*Retroviridae*	ssRNA	NC_001414	1	24	62.7 (18–185)	97.3 (88.0–100)

[a] GenBank accession number of the viral sequence closest in amino acid identity to sequences generated in this study.
[b] Number of samples out of 26 tested in which sequences mapping to each virus were detected (see Fig. 1).
[c] Number of sequence hits across all samples mapping to each virus.
[d] Length (amino acids) of sequence hits mapping to each virus across all samples (ranges in parentheses).
[e] Percent identity (amino acids) of sequence hits mapping to each virus across all samples (ranges in parentheses).

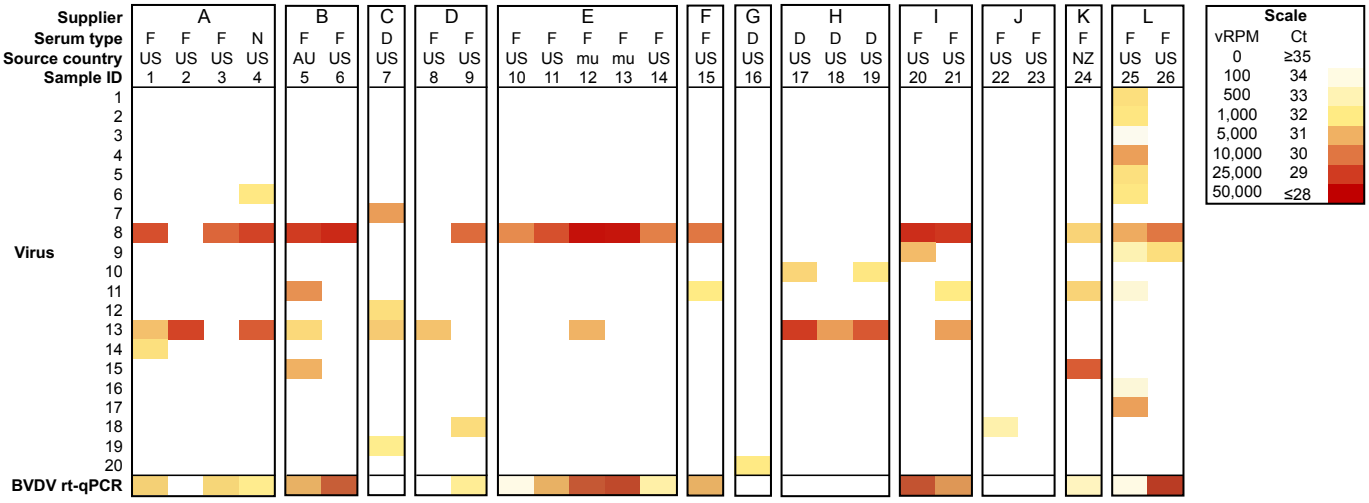

Fig. 1. Abundances of viruses in samples of commercial bovine serum inferred using metagenomics. Samples (1–26) came from 12 suppliers (A–L) and consisted of sera from fetal (F), newborn (N), or donor (D) animals. Sera were sourced from the USA (US), Australia (AU), New Zealand (NZ) or multiple unspecified countries (Mu). Virus numbers correspond to Table 1. Cells are colored in proportion to normalized reads (per million), calculated by mapping raw reads and contiguous sequences to the closest viral genomes in the GenBank database at the amino acid level. Real time quantitative PCR (rt-qPCR) data (ct values) for bovine viral diarrhea virus types 1 and 2 (virus numbers 8 and 9) are displayed at the bottom of the figure for comparison. The scale indicates viral reads per million from metagenomic data (vRPM, left) and cycle threshold (ct) values from rt-qPCR data (CT, right). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

sequence read depths ranged from zero to approximately 50,000 per million among the 11 manufacturers, with BVDV and the parvoviruses showing the highest read depths and the greatest variation in read depth (Fig. 1).

4. Discussion

Viral contamination of sera and other biological products remains a significant regulatory and economic problem [34]. The risks are not restricted to known viruses; they also apply to new and unexpected viruses, as illustrated by the case of porcine circovirus contamination of a licensed oral live-attenuated human rotavirus vaccine [35]. Each such instance of contamination and the ensuing regulatory response adds to the list of specific tests that biologics manufacturers are obliged to run on their products [10,17]. Metagenomics, which is "unbiased," provides a comprehensive assessment of the community of viruses in commercial sera, thus offering an attractive solution to the problem of ever-growing lists of adventitious viral targets for which products must be tested.

Metagenomic approaches detect not only known contaminants (e.g. BVDV) but also unknown viruses. Of the 32 viruses we detected, 17 were highly divergent from viruses present in the GenBank database (Table 1). This is perhaps not surprising considering the rapid rate of virus discovery, even in common domestic species such as cattle [36–38]. However, our finding of

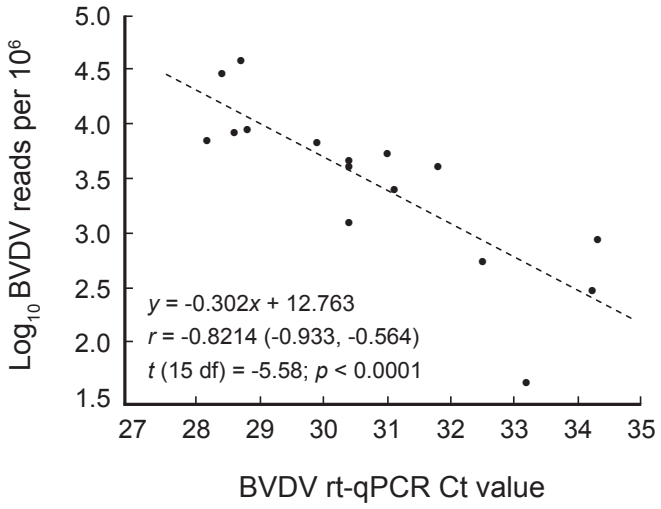

Fig. 2. Log-linear correlation between bovine viral diarrhea virus (types 1 and 2) abundance measured by cycle threshold (ct) values from reverse transcription real-time quantitative PCR (horizontal axis) and viral reads per million from metagenomic data (vertical axis) for 17 positive commercial bovine serum samples.

diverse novel viruses in commercial serum illustrates that relying on panels of specific tests for known viruses (although this may meet regulatory requirements) is inadequate for addressing the full scope of the biological problem. Metagenomics may provide an efficient and cost-effective solution [39].

We found that manufacturers vary widely in the level of contamination of their products. We do not know, however, whether this pattern would remain consistent over time. We tested only one lot of each product from each manufacturer. It is nonetheless noteworthy that several of the manufacturers whose products we tested market their sera using terms such as “high quality,” “contamination free,” “certified,” “ultra-clean” or other such terms implying a high degree of microbial purity. We observed no association between the use of such terms and actual levels of viral contamination. Similarly, assessments of reliability and reproducibility were beyond the scope of our study, but such assessments (within and between laboratories) would be necessary prior to industry-wide adoption of metagenomics for commercial testing [28].

Correspondence between rt-qPCR and metagenomics was high for BVDV, with the relationship between Ct values and read counts being log-linear. Among viral contaminants of bovine serum, BVDV has remained the most common [17], making this result noteworthy. Furthermore, the intercept of the line describing this relationship (Fig. 1) indicates that metagenomic methods such as ours are as sensitive as rt-qPCR, or perhaps even slightly more sensitive. Further work determining the sensitivity of metagenomics compared to PCR under different conditions is clearly needed, especially for situations in which viruses are identified on the basis of small numbers (even single) sequence reads [28].

Methods such as PCR rely on specific oligonucleotide priming to initiate polymerization, which renders PCR susceptible to “mismatches” in primer binding site sequences when used for testing of animal sera for adventitious viruses [20]. Because of the high abundance of BVDV in many of our samples, we were able to examine BVDV reads for any such primer binding site mismatches, but we found none. This exercise shows, however, that metagenomics may safeguard against minor viral genomic variation that could lead to PCR false negatives. Costs and ease of metagenomics are improving rapidly [40], such that these methods may soon compare favorably with more traditional diagnostic approaches such as multiplex PCR panels or technologies such as microarrays [41,42].

Our analyses cannot distinguish between viral nucleic acids and infectious viruses. The presence of viral nucleic acids may indicate contamination with infectious virus, or it may indicate inactivated virions or free viral genetic material. Nevertheless, even the presence of viral nucleic acids alone may be problematic, in that it can confound diagnostic tests, call into question the general purity of biologics, and indicate contamination at upstream stages of the manufacturing process. Also, our attempts to reconstruct individual viral genomes within samples were unsuccessful because of short read lengths and high variability in sequences. Commercial sera consist of pools of sera from many individual animals, such that the viruses we detected represent not distinct biological entities, but rather pluralities (“clouds”) of related sequences. These shortcomings will likely be resolved by sequencing technologies that generate longer read lengths from single nucleic acid molecules [43].

Our study focuses on bovine serum because of its importance for diagnostics and vaccine production. However, viral contamination can occur during the production of other biologics as well, leading to calls for new testing methodologies and accompanying validation standards [28,44,45]. For example, diverse viruses have been identified as contaminants of Chinese hamster ovary cells, which are widely used in biologics manufacturing [46]. Simian virus 40 (SV40; *Polyomaviridae*; *Polyomavirus*), porcine circoviruses (*Circoviridae*; *Circovirus*), and various bacteriophages and endogenous retroviruses have been detected in human vaccines, leading in some cases to significant regulatory actions [41,47]. Theiler's disease of horses has been iatrogenically transmitted to other horses by therapeutic administration of commercial horse plasma, causing hepatitis and fatal encephalopathy [48], and this occurred even before the causative viral agent (Theiler's disease-associated virus; *Flaviviridae*; *Pegivirus*) was discovered using metagenomic methods [49]. Thus, comprehensive testing methods such as those described herein would be useful for preventing the introduction of adventitious viruses, known or unknown, in diverse biologics and at various stages of their production and use.

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