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Multicenter benchmarking of short and long read wet lab protocols for clinical viral metagenomics

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ABSTRACT

Metagenomics is gradually being implemented for diagnosing infectious diseases. However, in-depth protocol comparisons for viral detection have been limited to individual sets of experimental workflows and laboratories. In this study, we present a benchmark of metagenomics protocols used in clinical diagnostic laboratories initiated by the European Society for Clinical Virology (ESCV) Network on NGS (ENNGS).

A mock viral reference panel was designed to mimic low biomass clinical specimens. The panel was used to assess the performance of twelve metagenomic wet lab protocols currently in use in the diagnostic laboratories of participating ENNGS member institutions. Both Illumina and Nanopore, shotgun and targeted capture probe protocols were included. Performance metrics sensitivity, specificity, and quantitative potential were assessed using a central bioinformatics pipeline.

Overall, viral pathogens with loads down to 10^4 copies/ml (corresponding to C_T values of 31 in our PCR assays) were detected by all the evaluated metagenomic wet lab protocols. In contrast, lower abundant mixed viruses of C_T values of 35 and higher were detected only by a minority of the protocols. Considering the reference panel as the gold standard, optimal thresholds to define a positive result were determined per protocol, based on the horizontal genome coverage. Implementing these thresholds, sensitivity and specificity of the protocols ranged from 67 to 100 % and 87 to 100 %, respectively.

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A variety of metagenomic protocols are currently in use in clinical diagnostic laboratories. Detection of low abundant viral pathogens and mixed infections remains a challenge, implying the need for standardization of metagenomic analysis for use in clinical settings.

1. Introduction

Pathogen-agnostic metagenomic sequencing has emerged as a universal diagnostic method for infectious diseases [1]. This methodology allows for identification and genomic characterization of pathogens without a priori knowledge of a suspected pathogen. This approach is gradually changing the way physicians diagnose and manage infectious diseases [2]. In addition, pan-viral respiratory pathogen surveillance has been launched using metagenomic approaches [3], enabling simultaneous tracking of all circulating viruses including potential novel ones, thus contributing to pandemic preparedness. The clinical utility of metagenomics in diagnosing idiopathic viral neurological syndromes has been reported in large prospective multi-center studies [4,5]. However, implementation of metagenomics routinely in patient care has lagged behind [2]. Hurdles for widespread introduction in diagnostic settings include the complex and time-consuming workflows, the technical challenge of low biomass clinical samples such as cerebrospinal fluid, and the complicated interpretation of contaminating sequences. In addition, universal reference standards that mimic the high complexity of patient samples, and standardized approaches to demonstrate assay validation, are lacking [2].

To date, reports on technical assessments of viral metagenomics protocols have been limited to individual sets of workflows and laboratories. Here, we present a benchmark study initiated by the European Society for Clinical Virology (ESCV) Network on NGS (ENNGS) including multiple metagenomic wet lab protocols used in clinical virology laboratories. A viral reference panel was designed to mimic low biomass clinical samples and used to assess the performance of twelve metagenomic wet lab protocols currently in use in diagnostic laboratories.

2. Methods

2.1. Construction of a reference panel

A viral metagenomic reference panel was designed to mimic low biomass clinical samples (e.g. respiratory swabs, cerebrospinal fluid) and their complexity, while reducing the number of environmental or cell culture-related sequences, to enable optimal sensitivity and specificity analyses. For this, twelve materials were prepared containing human cell free DNA (cfDNA, Twist pan-cancer reference standard set, 167 bp fragments with reduced methylation), spiked with synthetic viral sequences (both from Twist Bioscience, San Francisco, USA). These synthetic viral sequences covered >99.9 % of the viral genomes of SARS-CoV-2 B.1.1429 Epsilon strain USA/CA-CZB-12943/2020 (EPI_ISL_672365), influenza A virus strain A/California/07/2009 (H1N1, NC_026438), measles strain Ichinose-B95a (NC_001498.1), and enterovirus D68 Fermon strain (NC_038308.1), in non-overlapping fragments of maximal 5 kb with 50 bp gaps for biosafety reasons according to the manufacturer's policy. Viral sequences were mixed with several proportions of human cfDNA (90–99 % of weight, up to 400 pg per 100 µl), corresponding with final proportions of 10–1 % of viral nucleotides (down to 0.4 pg per 100 µl), based on the reported abundance in low biomass clinical samples [6–9]. The concentration of synthetic sequences was determined in triplicate by digital droplet PCR (BioRad QX200) and ranged from 10^4 to 10^7 copies/ml (cycle threshold, C_T values ranging from 24.4 to 31.1 in our assays [7,10]). A virus negative cfDNA control was included.

In addition, two dilutions (1:100 and 1:1000 in 0.1 Tris EDTA buffer) of ATCC Virome whole Virus Mix (MSA-2008™, ATCC, Manassas, USA) based on cultivated adenovirus (ADV) type F, cytomegalovirus (CMV), respiratory syncytial virus (RSV), influenza B virus, reovirus 3, and zika virus (C_T values from 27.8 to >40) were included in the panel. Viral loads of these dilutions were below the limit of quantification by the digital droplet PCR.

The panel was shipped to participants on dry ice, and receipt in good condition within 24 h was confirmed by all sites. Nucleic acid (NA) extraction of the two ATCC Virome Virus Mix dilutions was performed locally (Table 1). Subsequently, all NA underwent library preparation according to local protocols (see below). An overview of the study design is shown in Fig. 1.

2.2. Metagenomic protocols

In total, twelve metagenomic wet lab protocols were performed using the designed reference panel. The protocols were in use in the diagnostic laboratories of the participants. An overview of the protocol details and clinical use is shown in Table 1.

2.3. Bioinformatic analysis

Raw FASTQ datasets obtained in the participating laboratories were uploaded at a local website (hosted by the department of Medical Microbiology at the LUMC, Leiden) and analyzed using a previously validated [11–14] central bioinformatics pipeline to exclude variation introduced based on differences in bioinformatic analyses. Non-inferiority of the central pipeline was ensured through comparison with target virus results as obtained by the corresponding local pipelines, using a criterium of 100 % correspondence for qualitative detection of target viruses. Details on the local pipelines can be found in Suppl. Table 1 and a previously published ENNGS benchmark study of pipelines [14].

After central quality pre-processing and removal of human reads by mapping them to the human reference genome GRCh38 (https://www.ncbi.nlm.nih.gov/assembly/GCF_000001405.26/ using Bowtie2 [15] version 2.3.4), datasets were analyzed using Genome Detective [16] version 2.48 (accessed April – May 2023) as described previously [12]. Oxford Nanopore Technologies (ONT) datasets were subjected to QC using Genome Detective software. Genome Detective includes *de novo* assembly, and both nucleotide and amino acid-based classification in combination with a RefSeq/Swiss-Prot Uniref database [16]. Additional (off-target) viral classifications were confirmed by BLAST [17].

2.4. Performance metrics and statistical analyses

Both qualitative and quantitative performance of the protocols were analyzed based on horizontal genome coverage and sequence read counts. Sensitivity and specificity were calculated considering the reference panel as the gold standard; additional findings were considered false positives. Non-vertebrate viruses and endogenous retroviruses were excluded from analyses. Optimal thresholds to define a positive result were determined per protocol by varying the percentage of horizontal genome coverage, depicted in Receiver Operating Characteristic (ROC) curves and selecting the point on the curve with shortest Euclidian distance to the upper left corner (UL index, where coordinates (0,1) result in perfect classification). Target viruses up to PCR C_T-values of 35 were included in the analyses. Read counts were normalized for total read counts (number of reads after QC per million, RPM), and for genome size for quantitative comparison with PCR C_T-values using the formula: reads per kilobase per million (RPKM) = (number of reads

Table 1
Protocol details of the metagenomics methods analyzed.

Metagenomics protocol no.	1	2^	3	4	5	6	7	8	9	10	11	12
Technology	Illumina	Illumina	Illumina	Illumina	Illumina	Illumina	Illumina	Illumina	ONT	ONT	ONT	ONT
Clinical use	Experimental	Patient care	Patient care	Patient care	Patient care	Patient care	Experimental	Patient care	Experimental	Experimental	Experimental	Experimental
In house/ commercial	In house	In house	In house	In house	In house	In house	In house	Commercial	In house	In house	In house	In house
Centrifugation filtration[a]	No	No	No	No	2000 g 10 min, 0.45μm	No	15,000 g 3 min, no filtration	No	17,000 g 1 min, 0.8μm	17,000 g 1 min, 0.8μm	5000 g 5 min, 0.45μm	2000 g 10 min, 0.45μm
Nucleic acid extraction[a], **Input/output volume**	ELITe InGenius, ElitechGroup, 200 μl /100 μl	QIAsymphony DSP Virus / Pathogen Midi Kit; 400 μL / 110 μL	Qiagen EZ1 virus mini kit 400 μl/ 60 μl	MagnaPure 96 DNA and Viral NA, 200 μl /100 μl	bioMérieux EMAG, 1000 μl/50 μl	easyMag NucliSENS/ specific B, 400 μl/60 μl	Qiagen Viral RNA kit, 140 μl/100 μl	MagnaPure 96 DNA and Viral NA, 200 μl /100 μl	QIAamp Viral RNA mini kit (Qiagen), 140 μl/50 μl	QIAamp Viral RNA mini kit (Qiagen)	Roche High Pure RNA kit, 100 μl/30 μl	bioMérieux EMAG, 1000 μl/50 μl
Input volume for prep	DNA: 24 μl RNA: 24 μl	DNA : 5 ng RNA : 10–100ng	DNA: 26 μl RNA: 10 μl	DNA: 50 μl RNA: 8 μl	DNA: 5 μl RNA: 10 μl	DNA: 25 μl RNA : 11 μl	12 μl (in 3 reactions)	5 μl	2.8 μl	4 μl	22 μl (in 2 reactions)	DNA: 5 μl RNA: 10 μl
rRNA depletion	No	No	No (tissues only)	Yes	No	Yes	No	No	No	No	No	No
Human DNA depletion	No	No	No (tissues only)	No	No	No	No	No	No	No	No	No
Library prep. kit DNA	Illumina DNA prep (Nextera DNA flex), tagmentation	Nextera XT DNA Prep	NEBNext Ultra II DNA Prep	NEB Next Ultra II DNA	Nextera XT DNA Prep	TruSeq DNA	Combined DNA/RNA	Combined DNA/RNA: Twist Bioscience	Combined DNA/RNA: ONT Native Barcoding 96 V14	Combined DNA/RNA: ONT Native Barcoding 96 V14	Combined DNA/RNA: ONT PCR Barcoding Kit	ONT PCR Barcoding Kit
Library prep. kit RNA	See DNA prep, incl. ds-cDNA synthesis	TruSeq Stranded Total RNA	KAPA RNA HyperPrep	SMARTer Stranded Total RNA-Seq	Nextera XT DNA Prep	TruSeq Stranded Total RNA	Nextera XT					ONT PCR Barcoding Kit
Un/targeted	Untargeted	Untargeted	Untargeted	Untargeted	Untargeted	Untargeted	Untargeted	Targeted: capture probes	Untargeted	Untargeted	Untargeted	Untargeted
Random amplification cycles	12	12	8: DNA prep. 12: RNA prep.	12	12	12	30 (SISPA)	12	20 (WTA)	30 (SISPA)	30	45
Internal controls spiked	-	-	RNA: phage MS2	-	DNA: phage T1, RNA: phage MS2	DNA: phage T1, RNA: phage MS2	-	DNA: PhHV RNA: EAV	-	-	-	DNA: phage T1, RNA: phage MS2
Total reads (bases) sample (median), platform	0.6 M (56 M bases) MiSeq	15 M (1723 M bases)	8 M (610 M bases) NextSeq 550	13 M (1782 M bases) NextSeq500/ 2000	0.8 M (72 M bases) MiSeq	3.5 M (375 M bases)	1.1 M (104 M bases) NextSeq	0.9 M (121 M bases) NovaSeq6000	1.8 M (366 M bases) P2 Solo	3.9 M (1144 M bases) P2 Solo	0.1 M (43 M bases)	0.1 M (47 M bases) Flongle, GridION
Volume (concentration) per library added to sequencing pool prior to denaturation & dilution	120–200 μl (6 nM)	(1.5 nM)	0.9–13.5 μl (0.16–4.71 nM)	NextSeq 500: 40.5 μl (0.9 pM) NextSeq 2000: 1.9 μl (400 pM)	1.8 - 5 μl (6–20 pM)	2 μl (4 nM)	5 μl (4 nM)	3.8 μl (32 nM)	2.1 μl (9.5 nM)	2.1 μl (9.5 nM)	0.2–1.6 μl	2.5 μl (1.5–15 nM)

(*continued on next page*)

Table 1 (*continued*)

Metagenomics protocol no.	1	2^	3	4	5	6	7	8	9	10	11	12
References	Gradel et al [22] Bigi et al[23] Gradel et al [24]	Rodriguez et al [25]	Penner et al [26] Morfopoulou et al[27] Atkinson et al.2023 [18]	Alawi et al [28]	Kufner et al. [5]	Narat et al [29] Vallet et al [30] Garzaro[31] and Aboudar et al[32]	Cinek et al [33]	Mourik et al [19] Carbo et al [11] Reyes et al [34] De Kleine et al [35]	Vanmechelen et al[36]	Wollants et al [37] Vanmechelen et al[38]	Van Boheemen et al.[6]	Pichler et al [39]

[a] pre-nucleic acid extraction enrichment was applied to the ATCC Virome Mix dilutions containing whole viruses. WTA; whole transcriptome amplification, SISPA; sequence-independent, single-primer amplification. NA; not available.

mapped to the virus genome * 10^6) / (total number of reads * length of the genome in kb) [12]. In case of separate RNA and DNA libraries, given the variability in size, total read counts of the corresponding separate libraries were used.

3. Results

3.1. Metagenomic wet lab protocols

The designed low biomass reference panel mimicking patient samples was sent to ten diagnostic laboratories. In total, twelve different viral metagenomic protocols were in use in the participating laboratories. Protocol details and clinical use are shown in Table 1. Eight Illumina and four ONT protocols were included. One of the methods targeted vertebrate viruses by probe hybridization, all other methods were untargeted. Most Illumina protocols were used for patient care, while all ONT protocols had an experimental status. Enrichment before nucleic acid extraction by filtration was used in all ONT protocols, but only in one of the Illumina protocols evaluated. Separate DNA and RNA libraries, as opposed to combined libraries, were prepared in 6/8 Illumina protocols in contrast to 1/4 ONT protocols. None of the protocols made use of depletion of human CpG methylated DNA during library preparation. Two protocols depleted ribosomal RNA. Random amplification of 20 cycles or more was used in all ONT protocols, and one Illumina protocol (see Table 1), including sequence-independent, single-primer amplification (SISPA). The median sequence read counts generated per sample ranged from 0.6 M (56 M bases) to 15 M (1723 M bases) for Illumina protocols, and from 0.1 M (4.3 M bases) to 3.9 M (1144 M bases) for ONT protocols.

3.2. Detection of viral pathogens

Locally obtained FASTQ files were sent to the coordinating site for bioinformatic analyses. Performance of the metagenomic protocols was analyzed using a validated central pipeline that enabled processing of both short and long sequence reads. Overall, comparison of the results obtained by the central and local pipelines confirmed non-inferiority of the central pipeline in relation to the local pipelines as part of the overall local workflow for qualitative detection (Suppl. Table 1).

Qualitative detection of target viruses was first analyzed using the absolute, unnormalized data, representing the practical performance of the protocols with their corresponding platforms. A primary outcome parameter was selected that facilitated optimal comparison of short and long read protocols: the coverage percentage of the target virus genomes (% horizontal genome coverage, see Table 2). No thresholds for defining a positive result were used; that is, qualitative detection results by horizontal genome coverage and unnormalized read counts were therefore equivalent.

Using the central pipeline, all protocols (12/12) resulted in 100 % qualitative detection of the synthetic viral sequences spiked in up to 99 % human background sequences (viral loads 10^4–10^7 copies/ml, C_T values 24–31). Coverages of the viral genomes were consistently 100 % for viral loads of 10 [6] copies/ml or higher (C_T values of 24–26) when using the Illumina protocols, and ranged from 29 % to 100 % for the ONT protocols. Viral sequences with C_T values of 30–31 resulted in overall coverage of genomes of 95–100 % for four of the Illumina protocols, coverage by the remaining Illumina and ONT protocols was lower.

Viruses present in the ATCC Virome whole Virus Mix up to C_T values of 31 were detected by all Illumina protocols and 3/4 ONT protocols. Viruses in the mix with C_T values of 33 to 35 were detected by 6 to 7/12 of the methods.

As secondary outcome measure, normalized read counts were compared to study the efficiency of the protocols with regard to sequencing target virus genomes in relation to overall sequences generated. Detection of target viruses based on normalized read counts

(RPM) is shown in Table 3. Two of the four ONT protocols resulted in total read counts of median 1.0 M and higher (Table 1). Detection based on RPM varied significantly among Illumina protocols and ONT protocols in a pattern distinct from detection based on genome coverage presented in Table 2. Highest RPM counts were obtained using the virus probe targeted protocol (#8, up to 0.9 M RPM), the Illumina protocol with separate DNA and RNA libraries and rRNA depletion (#6, up to 0.6 M RPM) and the ONT SISPA protocol (#10, up to 0.9 M RPM, though less consistent).

The correlation between sequence read counts (RPKM) and viral loads (copies/ml) ranged from 0.41 for the targeted protocol (#11) indicating probe saturation, to 0.94 (#3, Pearson, Fig. 2). Illumina protocols generally had higher correlation coefficients than ONT protocols, with or without SISPA.

3.3. Sensitivity, specificity and ROC curves

Sensitivity and specificity were calculated considering the reference panel as the gold standard; other detected viral sequences were considered false positives, and non-vertebrate viruses and endogenous retroviruses were excluded from analyses. Viral sequences detected by the central bioinformatics pipeline are listed per protocol in Suppl. Table 2, at species level and with their percentage of genomes covered. To enable comparison of the specificity, optimal thresholds to define a positive result were determined per protocol. Receiver Operating Characteristic (ROC) curves were generated by varying the threshold for the horizontal genome coverage in percentages, for all protocols (Fig. 3). When considering the thresholds that resulted in optimal performance for each protocol (corresponding with the point on the curve with the shortest distance to the upper left corner (0,1), sensitivity varied from 67 % to 100 % (see legend of Fig. 3). Specificity varied from 87 % to 100 %. Sensitivities and specificities of 95 % and higher were obtained by four protocols: three Illumina (#1, #7, #8) and one ONT protocol (# 9).

A heatmap of the additional viral findings without taking into account these thresholds is shown in Table 4, including the number of positive samples per species, and the maximum horizontal genome coverage in case of multiple hits per species. Additional findings could be classified as target virus contaminants (reference panel sequences), and off-target findings. The latter were mainly categorized as protocol specific (multiple samples with a single viral species hit within one protocol) but also shared off-target viruses (alphapolyomaviruses, gammapapillomaviruses) were detected by multiple protocols. On-target contaminants were slightly more often detected when using the most sensitive ONT protocols (#9 and #10). It cannot be excluded that the papillomaviruses and polyomaviruses were actually present in low concentration in the human background cfDNA originating from plasma.

4. Discussion

To our knowledge, this is the largest multicenter multinational in-depth cross-platform comparison of metagenomic wet lab protocols for viral pathogen detection reported to date. A low biomass reference panel containing 400 pg total nucleic acids per sample, below the common input recommended by manufacturers of routine library prep kits, was designed to mimic clinical sample types most commonly requested for metagenomics (cerebrospinal fluid) and most relevant for pandemic preparedness (respiratory swabs).

When introducing optimal per protocol thresholds, sensitivity of 90 % and higher was accomplished using the majority of the protocols, and 10/12 protocols resulted in specificities of 95–100 %. The protocols all reported correct qualitative results down to PCR C_T values of 31, suggesting that the performances of protocols were acceptable for clinical and surveillance settings using low biomass samples. However, the detection of contaminating sequences even in samples of a reference panel probably corresponded to ambient lab contamination, which remains a challenge for clinical metagenomic sequencing. Indeed, both intra- and inter-experimental contamination are common confounders in clinical metagenomics. The targeted protocol had the largest number of additional findings when no threshold was taken into account. While the central pipeline may have detected additional hits potentially due to contamination, most local bioinformatics protocols included specific controls and thresholds for defining a true positive result, excluding reporting of other findings. In addition, some of the participating

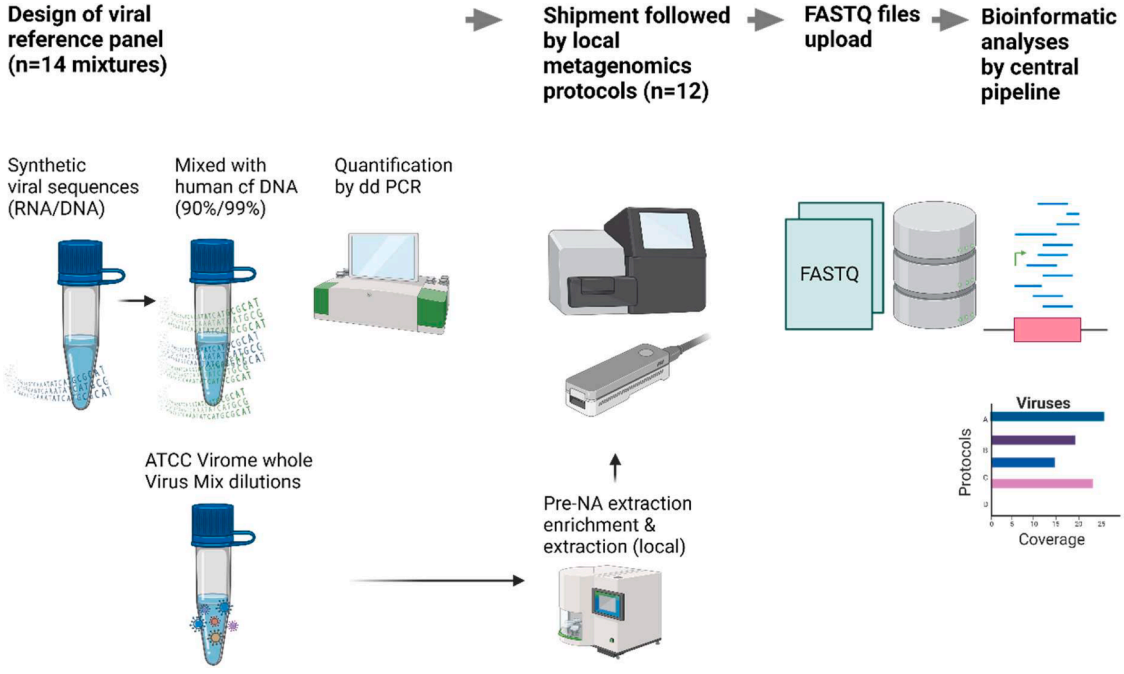


Fig. 1. Overview of the study design. A mock virus reference panel was constructed of mixtures of synthetic virus sequences in human cell free DNA, and Virome whole Virus Mix dilutions. The panel was shipped to participating laboratories and sequenced by local metagenomic Illumina and Oxford Nanopore Technologies (ONT) protocols. Locally obtained raw FASTQ files were uploaded and analysed using a central bioinformatic pipeline. Created using Biorender.

Table 2

Detection of target viruses using absolute, unnormalized data, representing the practical performance of the protocols with their corresponding platforms. The coverage percentage of the target virus genomes is depicted, enabling comparison of short and long read protocols. No thresholds for defining a positive result were used.

					Metagenomics protocol no. & coverage (%)											
					1	2	3	4	5	6	7	8	9	10	11	12
					Illumina								ONT			
		c/ml	Ct value	% viral	Separate DNA and RNA libraries					Combined DNA/RNA library						Separate
								rRNA depletion		rRNA depletion	SISPA 30 cycles	Viral probe targeted	20 cycles	SISPA 30 cycles	30 cycles	45 cycles
SS1	Mumps	10^6	25.6	10	100	100	100	100	100	100	100	100	95	100	98	29
SS2	Mumps	10^4	30.8	10	89	78	69	43	14	99	95	100	89	53	60	4
SS3	Mumps	10^4	31.1	1	95	9	35	38	57	99	96	100	86	51	80	2
SS4	Inf-A, H1N1	10^6	24.7	10	100	100	100	100	100	100	100	100	82	84	84	71
SS5	Inf-A, H1N1	10^4	30.4	10	65	80	74	28	10	99	96	99	60	25	61	15
SS6	Inf-A, H1N1	10^4	30.0	1	67	79	73	45	51	100	90	100	67	60	68	17
SS7	EV-D68	10^7	25.0	10	100	100	100	100	93	100	100	100	99	99	99	96
SS8	EV-D68	10^5	31.0	10	96	85	NA	90	81	100	98	100	95	95	74	11
SS9	EV-D68	10^5	31.0	1	NA	4	57	83	52	100	100	99	92	91	72	7
SS10	Inf-A, H1N1	10^6	24.9	10	100	100	100	100	100	100	100	100	78	91	91	77
	SARS-CoV-2	10^4	29.9	1	99	78	27	24	26	99	93	100	77	60	43	83
SS11	Inf-A, H1N1	10^4	30.5	1	98	41	45	39	23	99	95	100	75	46	68	10
	SARS-CoV-2	10^6	24.4	10	100	100	100	100	100	100	100	100	99	100	98	60
S13 Virome whole									Filtration				Filtration	Filtration	Filtration	Filtration
virus mix 1:100	HCMV		30.4		91	15	89	90	62	89	16	90	66	4	0	11
	HAdV-F		27.7		100	3	100	100	59	96	20	100	67	9	4	6
	Inf-B		33.3		40	0	6	0	0	0	4	92	33	15	0	18
	Reovirus-3		NA		98	5	10	14	19	65	6	99	88	0	0	0
	RSV		>40		2	0	0	0	0	0	1*	9	2*	0	0	0
	Zika virus		36.3		24	0	3	7	0	0	0	64	0	8	0	0
S14 Virome whole	HCMV		31.2		70	1	88	88	9	45	2	90	72	66	0	56
virus mix 1:1000	HAdV-F		30.7		63	0.3	100	97	14	9	3	99	71	1	0	0
	Inf-B		35.1		16	0	4	0	0	0	13	67	31	12	0	0
	Reovirus-3		NA		96	0	0	4	2	0	0	100	73	28	0	0
	RSV		>40		0	0	1	0	0	0	0	4	0	0	0	0
	Zika virus		36.2		0	0	0	2	0	0	6	26	3	0	0	0

Legend: 100 %, 80 %, 60 %, 40 %, 20 %, 10 %, 0 %

NA; not analysed

* classification/specificity BLAST confirmed

Table 3

Normalized read counts (RPM) for the target virus genomes in the panel, enabling comparison of efficiency of the protocols with regard to sequencing target virus genomes in relation to overall sequences generated. No thresholds for defining a positive result were used.

					Metagenomics protocol no. & RPM											
					1	2	3	4	5	6	7	8	9	10	11	12
					Illumina								ONT			
		c/ml	Ct value	% viral	Separate DNA and RNA libraries					Combined DNA/RNA library						Separate
								rRNA depletion		rRNA depletion	SISPA 30 cycles	Viral probe targeted	20 cycles	SISPA 30 cycles	30 cycles	45 cycles
SS1	Mumps	10^6	25.6	10	62080	827	4040	4515	5030	85273	152598	971014	5833	901959	46540	4755
SS2	Mumps	10^4	30.8	10	7302	18	3728	328	13	2654	6264	872159	1059	85726	2421	1180
SS3	Mumps	10^4	31.1	1	8184	1	676	199	963	3837	8100	877443	1140	21382	1200	1263
SS4	Inf-A, H1N1	10^6	24.7	10	45405	816	5241	4494	5474	66414	203639	976550	16741	72045	1234	192241
SS5	Inf-A, H1N1	10^4	30.4	10	396	12	9754	122	148	22788	10266	963562	7654	20343	631	110
SS6	Inf-A, H1N1	10^4	30.0	1	858	26	1169	182	108	47341	7588	958678	3858	8016	397	120
SS7	EV-D68	10^7	25.0	10	93008	430	17372	11280	7265	654953	259123	976777	15522	149771	65311	165705
SS8	EV-D68	10^5	31.0	10	5691	44	NA	582	240	38218	11957	907168	7159	112256	1737	5172
SS9	EV-D68	10^5	31.0	1	NA	0.1	1244	599	218	30018	13961	860338	2328	45223	2959	624
SS10	Inf-A, H1N1	10^6	24.9	10	52721	2313	662	5472	15635	348451	231861	974911	12528	77033	23262	165500
	SARS-CoV-2	10^4	29.9	1	3631	74	144	171	335	10053	4490	2357	109	2594	1480	2932
SS11	Inf-A, H1N1	10^4	30.5	1	1273	7	145	80	2725	7587	4820	151717	437	663	649	671
	SARS-CoV-2	10^6	24.4	10	214726	694	6386	6346	17376	534792	173390	819167	1070	27243	66387	89932
S13 Virome whole									Filtration				Filtration	Filtration	Filtration	Filtration
virus mix 1:100	HCMV		30.4		168982	5609	26549	40932	10430	20846	1029	498210	16690	1475	0	9733
	HAdV-F		27.7		10592	98	1878	1728	1949	2488	162	24554	1171	242	12	173
	Inf-B		33.3		5	0	0.1	0	0	0	1	315	1	49	0	467
	Reovirus-3		NA		865	0.08	0.1	10	74	226	3	14613	219	0	0	0
	RSV		>40		2	0	0	0	0	0	1	50	0.5	0	0	0
	Zika virus		36.3		14	0	0.1	5	0	0	0	302	0	53	0	0
S14 Virome whole	HCMV		31.2		518215	67	14629	7525	871	1865	94	416733	2444	39	0	2063
virus mix 1:1000	HAdV-F		30.7		31112	9	2198	1144	200	33	29	3635	201	2	0	0
	Inf-B		35.1		5	0	0.04	0	0	0	6	116	1	3	0	0
	Reovirus-3		NA		404	0	0	0.3	1	0	0	3563	438	101	0	0
	RSV		>40		0	0	0.02	0	0	0	0	21	0	0	0	0
	Zika virus		36.2		0	0	0	1	0	0	30	101	0.3	0	0	0

Legend: 100000 RPM, 10000 RPM, 1000 RPM, 100 RPM, 10 RPM, 1 RPM

NA; not analysed

laboratories also performed confirmatory PCR. Besides, bioinformatics misclassification of sequencing reads was minimized in this study by excluding additional findings that were not confirmed by BLAST (see methods). The data presented illustrate efficient filtering out false positive findings when implementing protocol-specific thresholds for defining a positive result. Extraction methods may have a considerable impact on protocol performance. Lower viral load samples were included in the whole virus mixture without human background sequences, potentially affecting the efficiency of protocol steps and the clinical performance. Nonetheless, many of the undetected whole

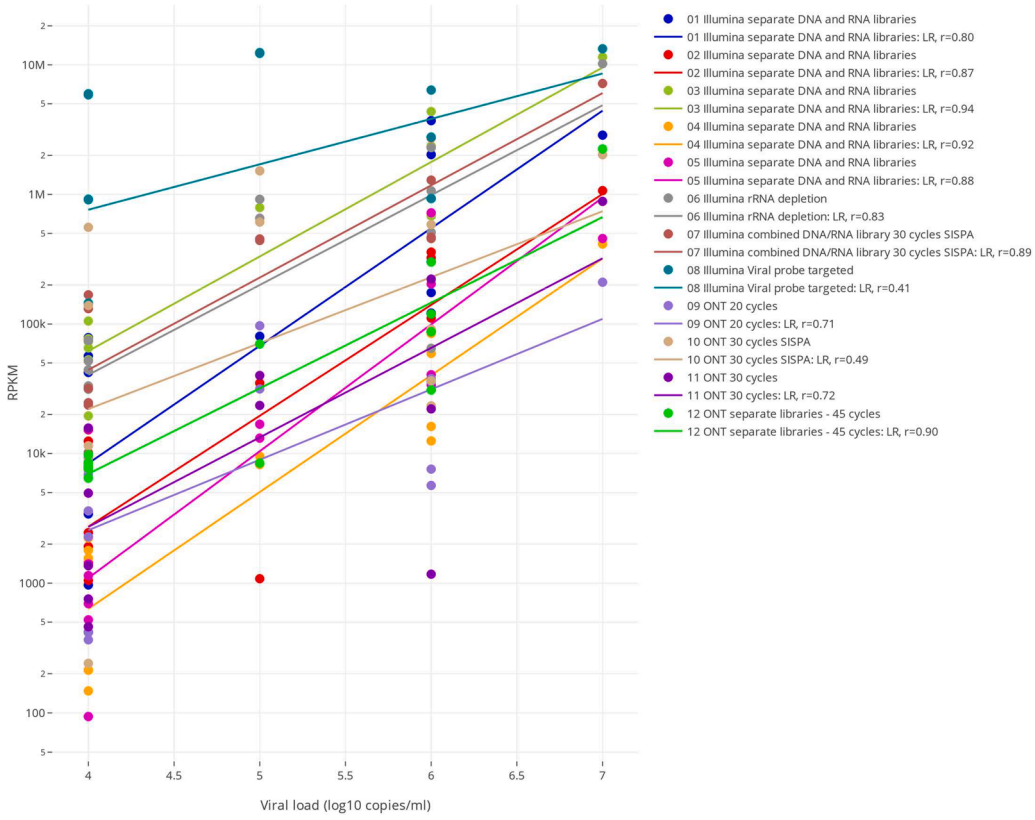


Fig. 2. Correlation between normalized sequence read counts (per kilobase per million, RPKM) and viral loads (copies/ml) for the protocols. Legend: LR; linear regression, r; Pearson's correlation coefficient, SISPA; sequence-independent single-primer amplification, ONT; Oxford Nanopore Technologies.

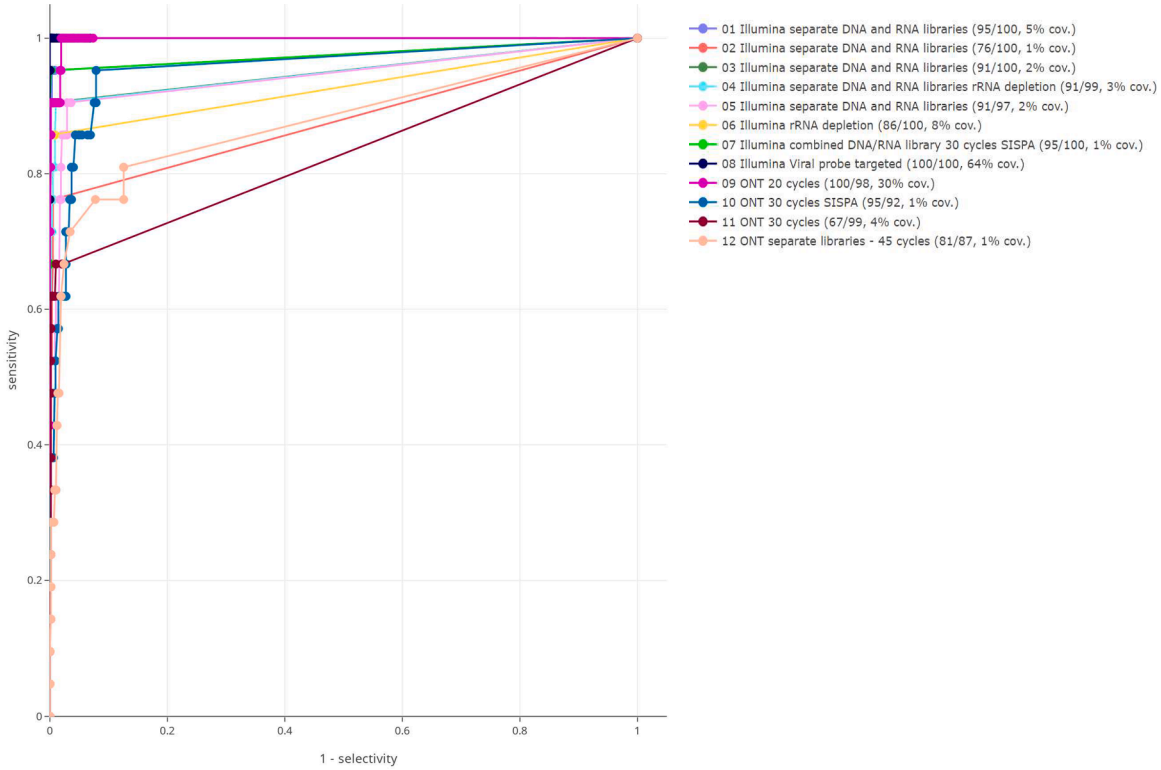
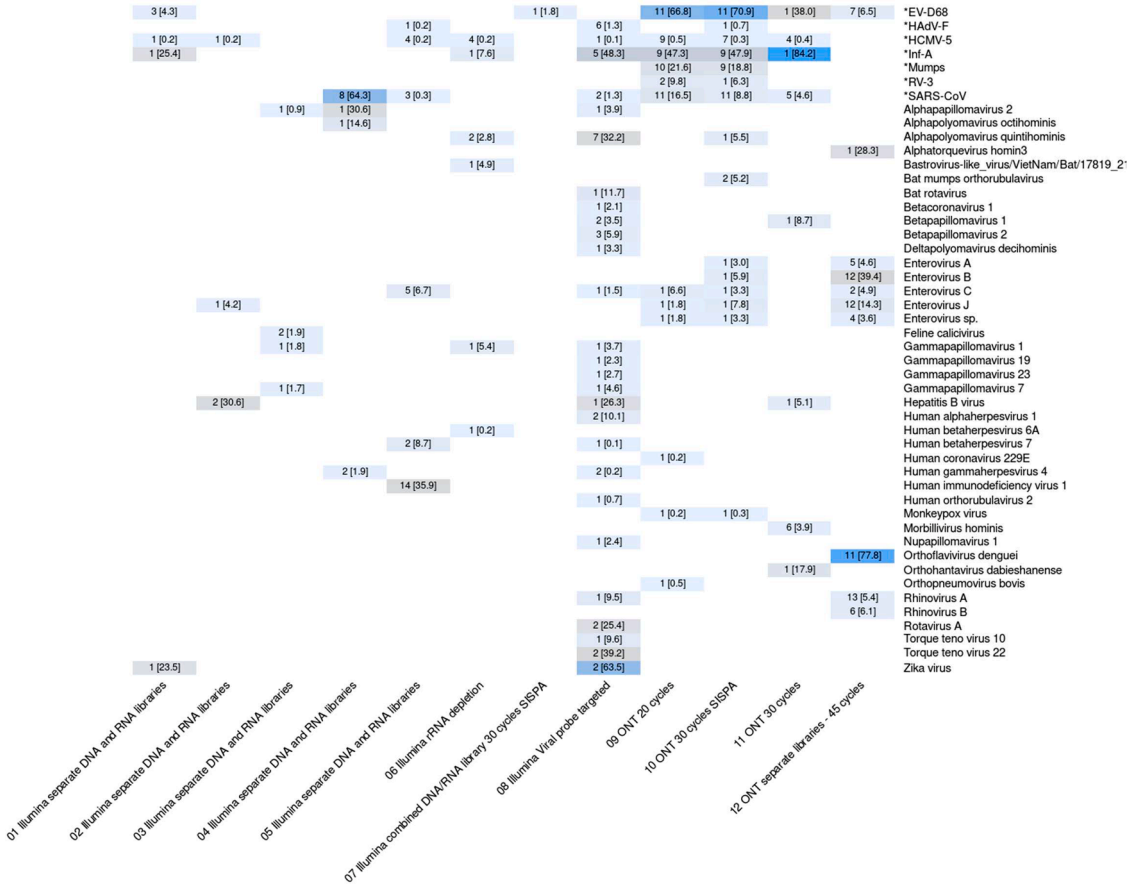


Fig. 3. Receiver operating characteristic (ROC) curves based on varying threshold of percentage genome covered for defining a positive result for the twelve metagenomic protocols. Viruses up to PCR CT-values of 35 were included in the analyses. Legend: protocol name, sensitivity/specificity (%), and optimal threshold for horizontal genome coverage (% cov.) at the optimal ROC point.

Table 4
Additional viral species findings per protocol, without using thresholds for defining a positive result. Depicted are the number of pos. samples and [max. horizontal coverage] per finding (coloring for maximum coverage value range: ≤33 %: pale blue, 33–66 %: grey, ≥66 %: blue). Pipeline classifications were confirmed by BLAST. Viruses marked with * are reference panel targets.



viruses were present at very low levels (CT values 33->40), close to or beyond the known limit of detection of some of the protocols [18,19].

A range of untargeted and targeted Illumina and ONT wet lab protocols were compared. Random amplification by SISPA (protocols #7, #10) resulted in higher normalized target read counts, however, this was not consistent for low viral load targets and did not result in improved horizontal genome coverage, indicating that amplification was not random over the entire viral genomes. The same phenomenon was seen for Illumina protocols that included either ribosomal RNA depletion or combined DNA and RNA libraries: the effect of higher coverages as compared to the other Illumina protocols was diminished when analyzing materials with low viral loads. It must be noted that the proportion of rRNA in this panel was low given a DNA only extraction that was used by Twist Bioscience to prepare the cfDNA, so the effect of an rRNA depletion step is less likely to be significant in this study setting. The hybridization targeted Illumina protocol (#8), however, consequently resulted in both higher genome coverage and normalized read counts.

This study has limitations. First, ideally, protocol comparisons are performed using clinical samples, but ring trials in general are limited by the available volume of clinical materials especially when assessing sterile sites such as CSF. We circumvented this limitation by using alternative materials mimicking clinical samples. Importantly, the reference panel enabled specificity analyses by providing a background that was relatively free from additional sequences. The panel provides a unique gold standard because it allows labeling of hundreds of viral species as true/false negative/positive, which would have been a tremendous effort to determine and quantitate by PCR in clinical samples. Metagenomic inter-laboratory comparisons and EQA schemes using cell-culture based or clinical materials are significantly challenged in defining a ground truth, and are therefore commonly forced to define a participant consensus truth as alternative. Second, by using nucleic acids as starting material to exclude potential effects of local nucleic acid extraction, pre-extraction enrichment steps [20,21] could only be studied partially. Moreover, the viral nucleic acids used were not fully representative of those occurring *in vivo*: transcriptional, splicing, and quasispecies variants were not represented. Background nucleic acids used did not comprise the complete host genome and did not mimic the size distribution of cell-free DNA/RNA, and bacterial or fungal nucleic acids were not present, however one would not expect a polymicrobial community in specimens from usually sterile sites such as CSF. We cannot exclude that this may have had impact on library preparation efficiency and bioinformatic analyses due to lower sample diversity. To assess the impact of the artificial distribution of viral nucleic acids on *de novo* assembly, read assignment analyses without genome reconstruction was performed by Centrifuge and as part of the analyses of the results of local pipelines (Suppl. Table 2). The results as obtained by the local pipeline were not inferior, suggesting that *de novo* assembly was not impactfully affected. Finally, the ssRNA and dsDNA reference pathogens included were aiming at pandemic preparedness and CSF syndromes, thus performance may not be entirely representative for other pathogens and all types of clinical materials. To summarize, this collaborative work provided unique insight into the current state of implementation of viral metagenomic protocols for pathogen detection

and the efficiency of a variety of wet lab protocol steps. In addition, we present a use case for a potential standardized bioinformatics validation approach using ROC curves with per protocol customized thresholds for calculation of specificity. This report aims to assist the implementation of viral metagenomics for pathogen detection in clinical diagnostic settings by providing insight into the efficacy of platforms and key protocol steps.

CRediT authorship contribution statement

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Declaration of competing interest

The authors declare to have no conflict of interest.

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Supplementary materials

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