

1 Proficiency testing of virus diagnostics based on bioinformatics analysis of simulated *in silico*
2 high-throughput sequencing datasets

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4 Annika Brinkmann^{a#}, Andreas Andrusch^a, Ariane Belka^b, Claudia Wylezich^b, Dirk Höper^b, Anne
5 Pohlmann^b, Thomas Nordahl Petersen^c, Pierrick Lucas^d, Yannick Blanchard^d, Anna Papa^e,
6 Angeliki Melidou^e, Bas B. Oude Munnink^f, Jelle Matthijnsens^g, Ward Deboutte^g, Richard J.
7 Ellis^h, Florian Hansmannⁱ, Wolfgang Baumgärtnerⁱ, Erhard van der Vries^j, Albert Osterhaus^k,
8 Cesare Camma^l, Iolanda Mangone^l, Alessio Lorusso^l, Maurilia Maracci^l, Alexandra Nunes^m,
9 Miguel Pinto^m, Vítor Borges^m, Annelies Kronemanⁿ, Dennis Schmitz^{f,n}, Victor Max Corman^o,
10 Christian Drosten^o, Terry C. Jones^{o,p}, Rene S. Hendriksen^c, Frank M. Aarestrup^c, Marion
11 Koopmans^f, Martin Beer^b, Andreas Nitsche^a

12

13 ^aRobert Koch Institute, Centre for Biological Threats and Special Pathogens 1, Berlin, Germany

14 ^bFriedrich-Loeffler-Institut, Institute of Diagnostic Virology, Greifswald–Insel Riems, Germany

15 ^cTechnical University of Denmark, National Food Institute, WHO Collaborating Center for
16 Antimicrobial Resistance in Foodborne Pathogens and Genomics and European Union Reference
17 Laboratory for Antimicrobial Resistance, Kgs. Lyngby, Denmark

18 ^dFrench Agency for Food, Environmental and Occupational Health & Safety, Laboratory of
19 Ploufragan, Unit of Viral Genetics and Biosafety, Ploufragan, France

20 ^eAristotle University of Thessaloniki, School of Medicine, Microbiology Department,
21 Thessaloniki, Greece

22 ^fErasmus Medical Centre, Department of Viroscience, Rotterdam, The Netherlands

23 ^gREGA Institute KU Leuven, Leuven, Belgium

24 ^hAnimal and Plant Health Agency, Addlestone, United Kingdom

25 ⁱUniversity of Veterinary Medicine Hannover, Department of Pathology, Hannover, Germany

26 ^jUniversity of Utrecht, Department of Infectious Diseases and immunity, Utrecht, The

27 Netherlands

28 ^kArtemis One Health Research Institute, Utrecht, the Netherlands

29 ^lIstituto Zooprofilattico Sperimentale dell’Abruzzo e Molise “G. Caporale”, National Reference

30 Center for Whole Genome Sequencing of microbial pathogens: database and bioinformatic

31 analysis, Teramo, Italy

32 ^mBioinformatics Unit, Department of Infectious Diseases, National Institute of Health (INSA),

33 Lisbon, Portugal

34 ⁿNational Institute for Public Health and the Environment, Bilthoven, The Netherlands

35 ^oInstitute of Virology, Charité-Universitätsmedizin Berlin, Berlin, Germany

36 ^pCenter for Pathogen Evolution, Department of Zoology, University of Cambridge, Cambridge,

37 United Kingdom

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39 Running Head: COMPARE *in silico* Virus Proficiency Test

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41 **# Address correspondence to Annika Brinkmann, BrinkmannA@rki.de**

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46

47 **Abstract**

48 Quality management and independent assessment of high-throughput sequencing-based virus
49 diagnostics have not yet been established as a mandatory approach for ensuring comparable
50 results. Sensitivity and specificity of viral high-throughput sequence data analysis are highly
51 affected by bioinformatics processing, using publicly available and custom tools and databases,
52 and differ widely between individuals and institutions.

53 Here, we present the results of the COMPARE (COllaborative Management Platform for
54 detection and Analyses of [Re-] emerging and foodborne outbreaks in Europe) *in silico* virus
55 proficiency test. An artificial, simulated *in silico* dataset of Illumina HiSeq sequences was
56 provided to 13 different European institutes for bioinformatics analysis towards the identification
57 of viral pathogens in high-throughput sequence data. Comparison of the participants' analyses
58 shows that the use of different tools, programs, and databases for bioinformatics analyses can
59 impact the correct identification of viral sequences from a simple dataset. The identification of
60 slightly mutated and highly divergent virus genomes has been identified as being most
61 challenging: Furthermore, the interpretation of the results together with a fictitious case report by
62 the participants showed that in addition to the bioinformatics analysis, the virological evaluation
63 of the results can be important in clinical settings.

64 External quality assessment and proficiency testing should become an important part of
65 validating high-throughput sequencing-based virus diagnostics and could improve harmonization,
66 comparability, and reproducibility of results. Similar to what is established for conventional
67 laboratory tests like PCR, there is a need for the establishment of international proficiency testing
68 for bioinformatics pipelines and interpretation of such results.

69

70 **Introduction**

71 High-throughput sequencing (HTS) has become increasingly important for virus diagnostic in
72 human and veterinary clinical settings and for disease outbreak investigations (1-3). Since the
73 introduction of the first HTS platform only about one decade ago, sequencing quality and output
74 have been increasing exponentially, combined with continuing decreased costs per base. Thus,
75 HTS has become a standard method for molecular diagnostics in many virological laboratories.
76 The relatively unbiased approach of HTS not only enables the screening of clinical samples for
77 common and expected viruses, but also allows an open view without preconceptions about which
78 virus might be present. This approach has led to the discovery of novel viruses in clinical
79 samples, such as Bas-Congo virus associated with hemorrhagic fever outbreaks in Central Africa
80 (2), Lujo arenavirus in southern Africa (3) and Bornavirus as the causative agent of several
81 cases of encephalitis with fatal outcome in Germany (4). Considering the potential of HTS to
82 complement or even replace existing 'gold-standard' diagnostic approaches such as polymerase
83 chain reaction (PCR) and qPCR, quality assessment (QA) and accreditation processes need to be
84 established to ensure quality, harmonization, comparability and reproducibility of diagnostic
85 results. While the computational analysis of the immense amount of data produced requires
86 dedicated computational infrastructure, bioinformatics knowledge or software developed by (bio-
87) informaticians, the interpretation of the results also requires evaluation by an experienced
88 virologist or physician. In many cases, true positive results can be difficult to discern among large
89 numbers of false positives, or may be entirely missing from result sets due to false negative
90 results. Interpretation of results also requires knowledge of anomalies that may arise through
91 sequencing artefacts or contamination.

92 Proficiency testing (PT) is an external quality assessment (EQA) for evaluating and verifying
93 sequencing quality and reliability in HTS analyses. The pioneer in EQA and PT for infectious

94 disease applications of HTS has been the Global Microbial Identifier (GMI) initiative, which has
95 been organizing annual PT's since 2015, focusing on sequencing quality parameters including
96 detection of antimicrobial resistance gene, Multilocus sequence typing, and phylogenetic analysis
97 of defined bacterial strains ([https://www.globalmicrobialidentifier.org/workgroups/about-the-](https://www.globalmicrobialidentifier.org/workgroups/about-the-gmi-proficiency-tests)
98 [gmi-proficiency-tests](https://www.globalmicrobialidentifier.org/workgroups/about-the-gmi-proficiency-tests)) (5). Subsequently, the concept was similarly established regionally for
99 United States laboratories offered by the FDA (6, 7).

100 COMPARE (COllaborative Management Platform for detection and Analyses of (Re-) emerging
101 and foodborne outbreaks in Europe, (<http://www.compare-europe.eu/>) is a European Union-
102 funded programme with participation of institutions with hands-on experience in viral outbreak
103 investigation and with the vision to improve the identification of (novel) emerging diseases
104 through HTS technologies. One of the ambitious goals is to establish and enhance quality
105 management and quality assurance in HTS, including external assessment and inter-laboratory
106 comparison.

107 In this study, we present the results of the first global PT to assess bioinformatics analysis of
108 simulated *in silico* clinical HTS virus data offered by the COMPARE network. The viral
109 sequence dataset was accompanied with a fictitious case report to facilitate a more real scenario
110 to support the identification of the simulated virus included the dataset.

111

112

113 **Tools and programs for bioinformatics analysis**

114 Over the past years, numerous tools, programs, and ready-to-use workflows have been
115 established, making metagenomics sequence analyses accessible to scientists from all research
116 fields. Workflows for the typical analysis of HTS data and for the identification of viral
117 sequences are based on the same general tasks and tools, including quality trimming,

118 background/host subtraction, *de novo* assembly, and sequence alignment and annotation.
119 Sequence processing usually starts with obligatory quality assessment and trimming, using
120 programs like FastQC or Trimmomatic, including removal of technical and low-complexity
121 sequences or filtering of poor-quality reads (8, 9). Following these initial steps, many workflows
122 include the subtraction of background reads, e.g., host and bacteria, to reduce the total amount of
123 data and increase specificity, using tools such as BWA (Burrows-Wheeler Alignment Tool) or
124 Bowtie2 (10, 11). *De novo* assembly of HTS reads into longer, contiguous sequences (contigs),
125 followed by reference-based identification, has been shown to improve the sensitivity of
126 pathogen identification. Such analyses depend heavily on the use of assemblers, such as SPAdes
127 or VELVET, which make use of specific assembly algorithms, such as overlap-layout-consensus
128 graph or de Bruijn graph algorithms (12, 13). Alignment tools like BLAST, DIAMOND, Kraken,
129 and Usearch are among the most important components in the bioinformatics workflows for
130 pathogen identification and taxonomic assignment of viral sequences (14-17). As command-line
131 tools for HTS sequence require specific knowledge in bioinformatics, complete workflows and
132 pipeline approaches were developed, including ready-to-use web-based tools, such as RIEMS
133 (Reliable Information Extraction from Metagenomic Sequence datasets), PAIPline (PAIPline for
134 the Automatic Identification of Pathogens), Genome detective, and others (18-20). As the
135 COMPARE *in silico* PT focuses on comparing different tools and software programs for
136 bioinformatics analyses, an overview of frequently-used programs is given in Table 1. A more
137 extensive overview of virus metagenomics classification tools and pipelines published between
138 2010 and 2017 can be found at (<https://compare.cbs.dtu.dk/inventory#pipeline>).

139

140

141 **Methods**142 *Organization*

143 The virus PT was initiated by the COMPARE network and organized by the Robert Koch
144 Institute. Invitations to participate were free of charge for research groups experienced in
145 analyzing HTS datasets, and were announced through email and the COMPARE website.

146 Participants were asked to analyze an *in silico* HTS dataset, with the main goal being to identify
147 the viral reads with their bioinformatics tools and workflows of choice and to interpret the
148 obtained results including final diagnostic conclusions.

149 An artificial, simulated *in silico* dataset of >6 million single-end 150bp long Illumina HiSeq
150 sequences derived from viral genomes, human chromosomes and bacterial DNA was provided to
151 13 different European institutes for bioinformatics analysis towards the identification of viral
152 pathogens in high-throughput sequence data. In order to assess how different level of experience
153 and/or bioinformatics methodologies affects the outputs and interpretation, participants were
154 allowed to use their bioinformatics tools and workflows of choice. Participants were invited to
155 report the PT results via an online survey within eight weeks (from September 16, 2016 until
156 November 16, 2016). Overall results were anonymized by the organizers but each participant was
157 provided with the identifier for their own results.

158

159 *In silico HTS dataset*

160 The simulated *in silico* dataset consisted of a total of 6,339,908 reads (Table 2), based on a
161 single-end 150-bp Illumina HiSeq 2500 run with an empirical read quality score distribution of
162 Illumina-specific base substitutions. The artificial dataset was simulated with the ART program
163 (21). Sequences were generated from the Human Genome Reference Consortium Build38
164 (GRCh38, NCBI accession CM000663–CM000686), *Acinetobacter johnsonii* (NCBI accession

165 NZ_CP010350.1), *Propionibacterium acnes* (NCBI accession NZ_CP012647.1) and
166 *Staphylococcus epidermis* (NCBI accession NZ_CP009046.1). In addition to human and bacterial
167 reads, simulated viral sequences of four viruses, Torque teno virus (TTV; NCBI accession
168 NC_015783.1), human herpesvirus 1 (HSV-1; NCBI accession NC_001806.2), measles virus
169 (MeV; NCBI accession NC_001498.1) and a novel avian bornavirus (nABV; NCBI accession
170 JN014950.1) were included in different numbers and with different levels of similarity to known
171 viruses present in databases (Table 2). TTV and HSV-1 were included in the panel as the easiest
172 sequences to identify (with 1,917 and 2,000 reads respectively, and 100% nucleotide identity
173 with the reference sequences), followed by a slightly altered MeV (1,000 reads, with 82%
174 nucleotide identity to the reference genome) and, as the likely most difficult taxon, nABV (only
175 500 reads and 55% nucleotide identity to reference JN014950.1). The dataset has been uploaded
176 to the European Nucleotide Archive with the study accession number PRJEB32470.

177

178 *Participants*

179 Thirteen participants applied for the COMPARE virus PT and completed the survey within the
180 given timeframe. Participants were registered from Belgium (n = 1), Denmark (n = 1), France (n
181 = 1), Germany (n = 4), Greece (n = 1), Italy (n = 1), The Netherlands (n = 2), Portugal (n = 1) and
182 United Kingdom (n = 1). The 13 participants represented 13 different institutes or organizations.
183 Information about the participants' background is given in Table 4.

184

185 *Case report*

186 To simulate clinical relevance and to set the background for evaluation of the bioinformatics
187 results, the following fictitious case report was provided with the dataset:

188 *Recently, a 14-year-old boy from Berlin, Germany, was hospitalized with sudden blindness,*
189 *reduced consciousness and movement disorders. The patient's mother reported developmental*
190 *disorders starting one year ago, with concentration problems, uncontrolled fits of rage, overall*
191 *decreasing performance in school and occasional compulsive head nods. Unfortunately, the*
192 *patient had received neither medical examination nor treatment, but had attended psychological*
193 *treatment, assuming behavioral problems.*

194 *Magnet resonance tomography of the patient's brain showed white and gray matter lesions and*
195 *gliosis. Soon after hospitalization, the patient showed a persistent vegetative state and died.*

196 *A sample of the boy's brain tissue was sequenced using the Illumina HiSeq 2500 platform,*
197 *resulting in approximately 6 million single end reads of 150 bp each.*

198 This case of subacute sclerosing panencephalitis (SSPE) can be caused by a persistent infection
199 with a mutated MeV (22). However, the symptoms described could also be caused by HSV-1 and
200 borna-like viruses (4, 23).

201

202 *Reported PT results*

203 Results were collected using the Robert Koch Institute's online survey software VOXCO. The
204 survey contained 23 questions including general participant information and specifications about
205 the programs used, parameter settings, computer specifications as well as the final results of the
206 PT, including an evaluation of the case. The responses were collected as single or multiple
207 options from a multiple-choice questionnaire with additional free text for remarks and comments.

208

209 *Analysis of PT results*

210 The results were evaluated based on sensitivity (true positive rate i.e. fraction of true virus reads
211 that were identified), specificity and total time of the bioinformatics analysis (Table 3). The time

212 of analysis was evaluated based on the computational time only, without time for preparation and
213 discussion of the bioinformatics results. Correlation of the time of analysis with computer and
214 server specifications was only based on use of online analysis, personal computer, server and
215 high-performance virtual machine. Although the pathogen identification by HTS-related
216 metagenomics should naturally involve experienced qualified health professionals, participants
217 were dared to attempt an interpretation regardless of the background of the team performing
218 bioinformatics. In this context, no qualitative and quantitative scoring was performed in this part.

219

220 **Results**

221

222 *PT results*

223 The results of the PT were evaluated based on sensitivity, specificity, total turnaround time, and
224 interpretation of results (Table 3). HSV-1 was identified by all participants (Tables 3-4, Fig. 1).
225 For most of the participants, the identified read numbers for HSV-1 were complete or near
226 complete (actual HSV-1 read count = 2,000). One participant identified more reads of HSV-1
227 than present in the dataset (participant 7; 8,361 reads identified).

228 TTV (actual read count = 1917) and MeV were identified by all participants except for one
229 (participant 4) (Tables 3-4, Fig. 1). For TTV, the read numbers identified were complete or
230 almost complete for all participants, with the exception of participant 9 who was only able to
231 identify 29% of the TTV reads. For the mutated MeV (actual read count = 1000), seven out of 13
232 participants were able to identify complete or almost complete read numbers (participants 3, 5, 6,
233 8, 10, 11, 12), whereas five participants (participants 1, 2, 9, 13) identified only 21%, 46%, 49%
234 and 34% of the total number of 1000 reads, respectively (Table 3). Participant 4 was unable to

235 identify MeV and participant 7 assigned too many reads (1,411) as originating from the mutated
236 MeV.

237 The divergent nABV (actual read count = 500) proved to be the most challenging target and was
238 identified by only four of the participants (participants 3, 5, 6, 12) (Tables 3-4, Fig. 1). The
239 overall specificity for all bioinformatics workflows was high, with only participant 6 identifying
240 43 reads of a chordopoxvirus as a false positive result.

241

242 The total time of analysis ranged widely, from three hours (participant 1) to 216 hours (online
243 analysis of 15 hours with additional 201 hours of waiting time for server availability, participant
244 4) (Table 5). Most workflows were calculated on a server system; two participants used a
245 personal computer and two participants a virtual machine. One calculation was executed through
246 an external public server.

247 Most of the workflows used in the COMPARE virus PT were quite similar, with the same basic
248 tasks applied in different order (Fig. 2). Most workflows started with trimming and quality
249 filtering, then subtraction of background reads, assembly of remaining reads, and a final
250 reference-based viral read assignment (Fig. 1). Databases used were custom-made or full
251 databases from NCBI nt/nr GenBank (participants 1-4, 6-11, 13). Participants 5 and 12 used viral
252 sequences from NCBI GenBank only, while participant 7 also included a database for human
253 pathogenic viruses (ViPR) (<https://www.viprbrc.org/brc/home.spg?decorator=vipr>).

254 All groups were also asked to correlate the results based on the bioinformatics analysis with the
255 clinical symptoms described in the case report (Table 4). HSV-1 was suspected as the disease-
256 causing agent by three groups and MeV was identified by six groups. An MeV infection with
257 HSV-1 possibly affecting the course of disease was named by two groups. nAVB was interpreted
258 as the single causative agent by two groups.

259

260 **Discussion**

261 HTS-based virus diagnostics requires a complex multistep processing, including laboratory
262 preparation, assessment of quality of sequences produced, computationally challenging analytic
263 validation of sequence reads, and post-analytic interpretation of results. Therefore, not only
264 comprehensive technical skills, but also bioinformatic, biological, and medical knowledge are of
265 paramount importance for proper analyses of HTS data for virus diagnostics.

266 HTS data can comprise several hundred thousand to many millions of reads from a single
267 sequenced sample. Handling and analyzing such amounts of data pose computational challenges
268 and currently require know-how and expertise in bioinformatics. Depending on the laboratory
269 procedure, identification of viral reads from clinical metagenomics data is negatively affected by
270 low virus-to-host sequence ratios and high viral mutation rates, making reference-based sequence
271 assignments for highly divergent viruses challenging (24).

272 The *In silico* bioinformatics analysis of HTS data can be separated into an analytic and a post-
273 analytic step. The analytic step includes the processing of sequence reads with software tools or
274 scripts assembled into workflows and pipelines. The post-analytic step is the evaluation of the
275 results obtained from the bioinformatics analysis, regarding pathogen identification often
276 involving the interpretation by an experienced qualified health professional to correlate
277 bioinformatics results with the clinical and epidemiological patient information.

278 The bioinformatics analysis and the technical identification of viral reads from the HTS dataset,
279 was shown to have a decreasing success as sequences became more divergent from reference
280 strains, exemplified by MeV with 82% identity on nucleotide level to its closest relative and
281 nABV with just 52 % identity on nucleotide level to other bornaviruses, identified by only four of
282 the 13 participants. MeV and TTV were missed by participant 4 whose analysis was based on the

283 Kraken tool and an in-house workflow. Kraken is known to align sequence reads to the reference
284 sequences with a high specificity and low sensitivity, making the alignment of mutated and
285 divergent virus reads difficult (15). As Kraken uses a user-specific reference database the TTV
286 may have been absent from the custom database, since Kraken was also used by participant 7,
287 who was able to identify both MeV and TTV. It is noted that the use of different databases is an
288 obstacle in bioinformatics analysis of HTS data. So far, there were only unified, curated virus
289 reference databases for Influenza viruses (EpiFlu) (25), HIV (26) and human pathogenic viruses
290 (ViPR) (27). Recently, viral reference databases for bioinformatics analysis of HTS data have
291 been developed (<https://hive.biochemistry.gwu.edu/rvdb>), (<https://rvdb-prot.pasteur.fr/>) (28).
292 NCBI offers the most extensive collection of viral genomes, but the lack of curation and
293 verification of submitted sequences often leads to false positive and false negative results. To
294 overcome such problems, reference-independent tools for virus detection of HTS data have been
295 developed, also making the discovery of novel viruses feasible without any knowledge of the
296 reference genome (29). All of the participants who were able to identify the divergent nABV
297 used workflows based on protein alignment approaches, including BLASTx/p, USEARCH, or
298 DIAMOND, which are known to be highly sensitive (14, 17). The identification of such highly-
299 divergent viruses is still challenging and cannot be accomplished by workflows based on
300 nucleotide-only reference-based alignment approaches. DIAMOND (double index alignment of
301 next-generation sequencing data), which became available in 2015, was specifically designed for
302 such sensitive analysis of HTS data at the protein level, and is up to 20,000 times faster than
303 BLAST programs. Compared to other alignment tools which seem to have a trade-off between
304 speed and sensitivity, DIAMOND offers superior sensitivity for the detection of mutated and
305 divergent viral sequences (14). However, the detection of such highly divergent viral sequences
306 in patient samples is rare, and virus discovery is not a routine part of clinical virus diagnostics.

307 In terms of specificity, all workflows were highly specific, with only workflow 6 showing the
308 identification of a chordopoxvirus which was not present in the dataset. Such false positives, as
309 well as the excessive number of HSV-1 and MeV reads found by participant 7 (8,361 of 2,000
310 reads and 1,411 of 1,000 reads, respectively) can derive, for example, from low-complexity reads
311 in the dataset which are aligned to low-complexity or repetitive sequences of the viral reference
312 genomes, from inappropriate matching score limits during filtering, or inappropriate algorithm
313 parameters. Furthermore, custom databases and viral references from NCBI can include
314 sequences of human origin which can lead to false positive results, which in some cases can
315 result in the non-reporting of other matches due to default algorithm reporting limits.

316 The total time of all workflows differed widely from only three hours to 216 hours (15 hours for
317 the analysis and 201 hours waiting time for available servers). One of the fastest participants was
318 participant 1 who needed only 3 hours to perform the calculations on a scalable high-performance
319 national virtual machine, whereas the slowest workflow (participant 4; 216 hours) was calculated
320 on a personal computer, through an external public server where bioinformatics software jobs are
321 queued among many other users (Fig. 1, Table 6). However, participant 5 also performed analysis
322 on a notebook but within a much shorter time (26 hours). Overall, workflows exclusively
323 specified for virus detection or using only viral or refSeq databases did not clearly correlate with
324 faster workflow times compared to full metagenomics analyses. However, the specific
325 composition of each database was not provided. To finally evaluate the performance of each
326 bioinformatics workflow regarding the time of analysis, all workflows should be run on the same
327 computer system, but such standardization was not practical for this PT evaluation.

328 The COMPARE virus PT has further shown that analytic and post-analytic evaluation is both of
329 importance, as similar analytic results can be interpreted very differently, depending on the
330 analyzing participant. Unlike standard routine virus diagnostic approaches such as polymerase

331 chain reaction, where a medical hypothesis of relevance is tested either positive or negative, HTS
332 offers an extensive and largely unbiased catalogue of results. The etiological agent of a patient
333 sample can be masked by false positives, sequencing contaminants, commensal viruses of the
334 human virome, or viruses of yet unknown importance. Furthermore, the causative viral agent of a
335 disease may be present in very low read numbers because viral loads may be low, depending on
336 the timing of sampling and the sample matrix. RNA viruses, some of which are the most
337 pathogenic human viruses, usually have smaller genomes than DNA viruses (30, 31). Therefore,
338 low read numbers from an RNA virus might be dismissed, resulting in a false negative. To assess
339 sequencing results, some workflows and pipelines use cutoffs for read numbers so as to reduce
340 false positives, but may in the process make the detection of low read-number matches less
341 likely.

342 As the analysis of HTS data for virus diagnostics requires bioinformatics as well as virological
343 knowledge, the collaboration of both disciplines has been emphasized (32). Furthermore,
344 automated pipelines for HTS-based virus diagnostics with unbiased evaluation of pathogenicity
345 and relevance of the detected pathogen have been implemented, which can render analysis and
346 interpretation of HTS sequence results more harmonized (33).

347 A robust approach to viral diagnostics using HTS requires further refinement and validation. The
348 COMPARE *in silico* PT is limited by the low complexity of the simulated dataset. *In Vivo*
349 sequence datasets can consist of a high diversity of the background and microbiome of the host,
350 which further increases the difficulty to identify viral reads. Further proficiency schemes with *in*
351 *vivo* datasets and samples and wider collaboration are required to make progress. A second *in*
352 *silico* PT organized by the COMPARE network has focused on the interpretation of the
353 significance of food-borne pathogens in a simulated dataset (data not published). Again, the
354 interpretation of the results was shown to be one of the most diverse and critical points in HTS

355 data analysis. Furthermore, third-generation sequencing technologies, such as the MinION from
356 Oxford Nanopore Technologies, are becoming available in many laboratories and field settings
357 due to low cost and short sequencing times (34-36). However, analysis tools developed for
358 second-generation sequencing technologies, such as Illumina, may not be applicable for third-
359 generation sequencing data, due to the low sequencing accuracy of approximately 85 % and of
360 and the length of the sequences, which can be up to 2Mbp (37-39). Consequently, future PTs
361 should also include the use of third-generation sequencing technologies, as those are likely to
362 become part of future routine laboratory diagnostics.

363

364 **Conclusion**

365 The present availability of External Quality Assessment for HTS-based virus identification is
366 limited. The COMPARE *in silico* virus PT has shown that numerous tools and different
367 workflows are used for virus analysis of HTS data, and results of such workflows differ in
368 sensitivity and specificity. At the present time, there are no standard procedures for virome
369 analyses, and the sharing, comparing, and reliable production of results of such analyses are
370 difficult.

371 Finally, there is a clear need for creating updated and highly curated, publicly freely available
372 databases for harmonized identification of virus in virome datasets, as well as mechanisms for
373 conducting continuous ringtails to ensure the quality of virus diagnostic and characterization in
374 clinical diagnostic and public and veterinary health laboratories.

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378

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509 Figure legends

510

511 **FIG 1 Identified viral read numbers for Torque Teno virus (TTV), human herpesvirus**

512 **(HSV-1), measles virus (MeV), and new avian bornavirus (nABV) by participant**

513 **(numbered 1-13).**

514

515 **FIG 2 Simplified comparison of different bioinformatics workflows for virus identification**

516 **used in the COMPARE virus proficiency test**

517 **+ Human herpesvirus, + Torque teno virus; + Measles virus; + Avian bornavirus**

518

519 **TABLE 1 Tools and programs for analysis of HTS data used in the COMPARE Virus Proficiency Test, in alphabetical order**

Program	Application	Description/relevance for viral HTS	URL
BWA (10)	Alignment (nucleotide)	BWA (Burrows-Wheeler Alignment Tool) to align efficiently short sequencing reads against a large reference genome. Based on string matching with Burrows-Wheeler transform (BWT).	http://bio-bwa.sourceforge.net/
DIAMOND (14)	Alignment (protein)	Double-index alignment of NGS data. Shown to be up to 20,000 times faster than comparable programs, with high sensitivity.	http://ab.inf.uni-tuebingen.de/software/diamond/
FastQC (9)	Quality control, trimming	Generates base quality scores and sequence contents, sequence length distributions, identification of duplicate or overrepresented sequences, adapter, and k-mer contents.	https://www.bioinformatics.babraham.ac.uk/projects/fastqc/
Kmerfinder (40)	Taxonomic assignment	Online user interface also allows the prediction of human and vertebrate viruses.	https://cge.cbs.dtu.dk/services/KmerFinder/
Kraken (15)	Alignment (nucleotide)	Only uses exact alignments for its taxonomic classification with high speed and less computational requirements.	https://ccb.jhu.edu/software/kraken/
MetaPhlan	Taxonomic assignment	Metagenomic Phylogenetic Analysis is a tool for the taxonomic assignment of microbial communities. High accuracy and speed are supported by only high-confidence matches. Such approaches allow the assignment of 25,000 microbial reads per second but might fail with	https://bitbucket.org/biobakery/metaphlan2

		viral genomes which often lack common markers and genes.	
MGMapper (41)	Pipeline	Online tool for processing, assigning, and analyzing HTS sequences.	https://cge.cbs.dtu.dk/services/MGmapper/ , https://bitbucket.org/genomicepidemiology/mgmapper
MIRA	<i>De novo</i> assembly	Mimicking Intelligent Read Assembly, overlap-layout-consensus graph (OLC) assembler for metagenomics data from several sequencing platforms. Assembles the most as well as the largest contigs compared to other <i>de novo</i> assembly programs, as well as produces the highest number of contigs which could be assigned to a viral taxon.	https://sourceforge.net/projects/mira-assembler/
NCBI BLAST (16)	Alignment (nucleotide and protein)	Basic local alignment search tool. Offers very sensitive online and stand-alone alignments of nucleotides, translated nucleotides, and protein sequences.	https://blast.ncbi.nlm.nih.gov/Blast.cgi
One Codex (42)	Taxonomic assignment	Web-based data platform for k-mer based taxonomic classification. Very high degree of sensitivity and specificity, even when analyzing highly divergent and mutated sequences.	https://www.onecodex.com/
PAIPline (20)	Pipeline	Pipeline for metagenomic analysis of HTS data.	https://gitlab.com/rki_bioinformatics/pipeline

QUASR (43)	Pipeline	Combination of several R packages and external software for HTS read analysis. Part of the Bioconductor project.	http://www.bioconductor.org
RIEMS (18)	Pipeline	Pipeline for metagenomics sequence analysis, combining several established programs and tools for pathogen detection in one automated workflow. Separated into a workflow of accurate and fast “basic analysis” and a more sensitive “further analysis”.	https://www.fli.de/en/institutes/institute-of-diagnostic-virology-ivd/laboratories-working-groups/laboratory-for-ngs-and-microarray-diagnostics/
Skewer (44)	Quality control, trimming	Trimming of primer and adapter sequences focusing on the characteristics of paired-end and mate-pair reads. A statistical scheme based on quality values allows the accurate trimming of adapters with mismatches.	https://sourceforge.net/projects/skewer/
SNAP (45)	Alignment (nucleotide)	Up to 10 to 100 times faster than similar alignment programs but offers greater sensitivity due to richer error acceptance.	http://snap.cs.berkeley.edu/
SPAdes, MetaSPAdes (12)	<i>De novo</i> assembly	De Bruijn graph assembler. MetaSPAdes specifically addresses the challenges that arise with complex metagenomics data.	http://cab.spbu.ru/software/spades/
Taxonomer (46)	Taxonomic assignment	Web-based tool for nucleotide- and protein-based read assignment. User-friendly interactive result visualization. Based on exact k-mer matching	http://taxonomer.iobio.io/

		with low error tolerance. Speed up to ~32 million reads/minute. Furthermore, protein-based read identification offers the detection of divergent viral sequences but is based on exact k-mer matching without error allowance.	
Trimmomatic (8)	Quality control, trimming	Paired-end sequence reads can be cut from technical sequences as adapters, primers, or low-quality bases. Has been shown to improve considerably downstream analyses, for example <i>de novo</i> assembly (increasing contig size up to 77 %) and alignment (increasing alignment rates from 7 % to 78 %).	www.usadellab.org/cms/index.php?page=trimmomatic
USEARCH (17)	Alignment (protein)	Exceptionally high speed for protein or translated nucleotide read alignment. The sensitivity of USEARCH is comparable to the NCBI protein BLAST, but USEARCH is ~350 times faster.	https://www.drive5.com/usearch/
Velvet (13)	<i>De novo</i> assembly	Can be used for <i>de novo</i> assemblies of short HTS reads using the de Bruijn algorithm. <i>de novo</i> assembly using Velvet can be achieved in as little as 14 minutes.	https://www.ebi.ac.uk/~zerbino/velvet/

520

521

522 **TABLE 2 Composition of the simulated sequence dataset. Total number of reads are**523 **6,339,908.**

Organism	#Reads	Nucleotide sequence identity with reference (%)
Human	4,834,491	100
<i>Acinetobacter johnsonii</i>	500,000	100
<i>Propionibacterium acnes</i>	500,000	100
<i>Staphylococcus epidermidis</i>	500,000	100
Torque teno virus	1,917	100
Human herpesvirus 1	2,000	100
Measles virus	1,000	82
(Novel) Avian bornavirus	500	55

524

525 **TABLE 3 Sensitivity and specificity for identified reads of the COMPARE virus proficiency**526 **test. Participants were numbered randomly.**

	Torque teno virus	Human herpesvirus	Measles virus	Avian bornavirus	No false positive result	Time of analysis (h)
#1	1	0.99	0.21	0	√	3
#2	1	1.01	0.46	0	√	15.5
#3	0.96	0.96	1	1	√	60
#4	0	0.10	0	0	√	216
#5	1	0.98	1	1	√	26

#6	1	0.84	1	1	-	12
#7	0.94	4.00	1.41	0	√	6
#8	1	1.04	0.99	0	√	7
#9	0.29	0.84	0.49	0	√	5
#10	1	1	1	0	√	48
#11	1	1	1	0	√	14
#12	1	1	1.02	0.23	√	18
#13	1.02	0.90	0.34	0	√	48

527

528 **TABLE 4 Interpretation of bioinformatics results.** Abbreviations: TTV = Torque teno virus,

529 HSV-1 = human herpesvirus 1, MeV = measles virus, nABV = new avian bornavirus

	Results bioinformatics	Results diagnostics	Participants' background
#1	TTV, HSV-1, MeV	HSV-1	Bioinformatics
#2	TTV, HSV-1, MeV	HSV-1	Food & environmental health
#3	TTV, HSV-1, MeV, nABV	SSPE/HSV-1	Veterinarian, virology
#4	HSV-1	HSV-1	University, virology
#5	TTV, HSV-1, MeV, nABV	nABV	Virology
#6	TTV, HSV-1, MeV, nABV	nABV	Medical research
#7	TTV, HSV-1, MeV	SSPE	Animal and plant health
#8	TTV, HSV-1, MeV	SSPE	Veterinarian, virology
#9	TTV, HSV-1, MeV	SSPE	Public health
#10	TTV, HSV-1, MeV	SSPE	Public health
#11	TTV, HSV-1, MeV	SSPE	Public health and environment

#12 TTV, HSV-1, MeV, nABV	SSPE/HSV-1	Diagnostics, virology
#13 TTV, HSV-1, MeV	SSPE	Virology

530

531

532 **TABLE 5 Total time of computational analysis, maximum computer/server specifications,**
533 **and reference databases used.**

	Time of analysis (h)	Database	Operating system	CPU	CPU Mhz	RAM (GB)
#1	3	NCBI nt	UNIX Ubuntu 16.04	VM	VM	VM
#2	15.5	NCBI nt	LTS	56	1270	378
#3	60	NCBI nt/nr	CentOS 6	24	2400	64
#4	216	NCBI nt	Windows XP	intel core i5	2300	8
#5	26	NCBI viral db	OS X	2	na	na
#6	12	NCBI nr VIPR and	Ubuntu 14.04 BioLinux	32	2000	503
#7	6	NCBI nt	Ubuntu 14.04	8	3.6	16
#8	7	NCBI nt	CentOS 6.5	64	2300	250
#9	5	NCBI nr	Ubuntu 12.04.5	na	3800	50
#10	48	NCBI nt	CentOS 6.5	2 × AMD Opteron	2200	32
					VM,	VM,
#11	14	NCBI nt/nr	RHEL	VM, variable	variable	variable
#12	18	NCBI viral db	Linux Mint	Intel Xenon	6 × 2.67	25

28

X5650

Ghz

Ubuntu 14.04.4 2 × AMD Opteron 24 × 2.2

#13 48

NCBI nt

LTS

6174

GHz

128

534 db=database; na= not available; nr=non-redundant; nt=nucleotide; VM=virtual machine

535



