

1 **Metagenomic Next-generation Sequencing of Cerebrospinal Fluid for the**
2 **Diagnosis of Central Nervous System Infections: A Multicentre Prospective**
3 **Study**

4
5 Siyuan Fan MD^{1*}, Xiaojuan Wang MD^{2*}, Yafang Hu MD, PhD³, Jingping Shi MD⁴,
6 Yueli Zou MD⁵, Weili Zhao, MS⁶, Xiaodong Qiao MS⁶, Chunjuan Wang, MD⁷,
7 Jerome H. Chin, MD, PhD, MPH⁸, Lei Liu MD⁹, Lingzhi Qin MD², Shengnan Wang
8 MD³, Hongfang Li MB¹⁰, Wei Yue MD¹¹, Weihe Zhang, MD¹², Xiaohua Li MM¹³,
9 Ying Ge MD¹⁴, Honglong Wu MS^{15, 16}, Weijun Chen PhD^{17, 18}, Yongjun Li PhD¹⁹,
10 Tianjia Guan PhD²⁰, Shiyong Li MM²¹, Yihan Wu MD²², Gaoya Zhou MM²³, Zheng
11 Liu MD²⁴, Yushun Piao MD²⁵, Jianzhao Zhang MM²⁶, Changhong Ren MD²⁷, Li Cui,
12 MD, PhD²⁸, Caiyun Liu MD²⁸. Haitao Ren BS¹, Yanhuan Zhao BS¹, Shuo Feng MM²⁶,
13 Haishan Jiang MD³, Jiawei Wang MD⁹, Hui Bu MD⁵, Shougang Guo MD⁷, Bin Peng
14 MD¹, Liying Cui MD¹, Wei Li PhD², Hongzhi Guan MD¹.

15
16 ¹ Department of Neurology, Peking Union Medical College Hospital, Chinese
17 Academy of Medical Sciences and Peking Union Medical College, Beijing, China.

18 ² Department of Neurology, People's Hospital of Zhengzhou University, Henan
19 Provincial People's Hospital, Zhengzhou, China.

20 ³ Department of Neurology, Nanfang Hospital, Southern Medical University,
21 Guangzhou, China.

22 ⁴ Department of Neurology, Affiliated Nanjing Hospital of Nanjing Medical

23 University, Nanjing, China.

24 ⁵ Department of Neurology, The Second Hospital of Hebei Medical University,

25 Shijiazhuang, China.

26 ⁶ Department of Neurology, Affiliated Hospital of Chifeng University, Chifeng,

27 China.

28 ⁷ Department of Neurology, Shandong Provincial Hospital Affiliated to Shandong

29 University, Jinan, China.

30 ⁸ Department of Neurology, Division of Global Health, New York University Langone

31 Health, New York, The United States of America.

32 ⁹ Department of Neurology, Beijing Tongren Hospital, Capital Medical University,

33 Beijing, China.

34 ¹⁰ Department of Neurology, Affiliated Hospital of Jining Medical University, Jining,

35 China.

36 ¹¹ Department of Neurology, Tianjin Huanhu Hospital, Tianjin, China.

37 ¹² Department of Neurology, China–Japan Friendship Hospital, Beijing, China.

38 ¹³ Department of Paediatrics, Affiliated Hospital of Inner Mongolia Medical

39 University, Hohhot, China.

40 ¹⁴ Department of Infectious Diseases, Peking Union Medical College Hospital,

41 Chinese Academy of Medical Sciences and Peking Union Medical College, Beijing,

42 China.

43 ¹⁵ Tianjin Medical Laboratory, BGI-Tianjin, BGI-Shenzhen, Tianjin, China.

44 ¹⁶ Binhai Genomics Institute, BGI-Tianjin, BGI-Shenzhen, Tianjin, China.

45 ¹⁷ University of Chinese Academy of Sciences, Beijing, China.

46 ¹⁸ Key Laboratory of Genome Sciences and Information, Beijing Institute of

47 Genomics, Chinese Academy of Sciences, Beijing, China.

48 ¹⁹ BGI genomics, BGI-Shenzhen, Shenzhen, China.

49 ²⁰ School of Public Health, Chinese Academy of Medical Sciences and Peking Union

50 Medical College, Beijing, China.

51 ²¹ Department of Neurology, North China University of Science and Technology,

52 Tangshan, China.

53 ²² Department of Neurology, Inner Mongolia People's Hospital, Hohhot, China.

54 ²³ Department of Neurology, Brain Hospital of Hunan Province, Changsha, China.

55 ²⁴ Department of Neurology, Xuanwu Hospital, Capital Medical University, Beijing,

56 China.

57 ²⁵ Department of Neurology, Shenzhen Baoan Shiyan People's Hospital, Shenzhen,

58 China.

59 ²⁶ Department of Neurology, Capital Institute of Paediatrics, Beijing, China.

60 ²⁷ Department of Neurology, Beijing Children's Hospital, Capital Medical University,

61 Beijing, China.

62 ²⁸ Department of Neurology and Neuroscience Centre, the First Hospital of Jilin

63 University, Changchun, China.

64

65 * Siyuan Fan and Xiaojuan Wang have contributed equally to this work.

66

67 Corresponding author: Hongzhi Guan.

68 Department of Neurology, Peking Union Medical College Hospital, Chinese Academy

69 of Medical Sciences and Peking Union Medical College, Beijing 100730, China

70 Telephone Number: (0086) 10-69156371

71 Fax Number: (0086) 10-69156371

72 Email: guanhz@263.net

73

74 Corresponding author: Wei Li.

75 Department of Neurology, People's Hospital of Zhengzhou University, Henan

76 Provincial People's Hospital, Zhengzhou 450003, China.

77 Telephone Number: (0086) 13939061069

78 Fax Number: (0086) 371-65897739

79 Email: liwein@zzu.edu.cn

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85 **ABSTRACT**

86 **Background:** Infectious encephalitis and meningitis are often treated empirically
87 without identification of the causative pathogen. Metagenomic next-generation
88 sequencing (mNGS) is a high throughput technology that enables the detection of
89 pathogens independent of prior clinical or laboratory information.

90 **Methods:** The present study was a multicentre prospective evaluation of mNGS of
91 cerebrospinal fluid (CSF) for the diagnosis of suspected central nervous system
92 infections.

93 **Results:** A total of 276 patients were enrolled in this study between Jan 1, 2017 and
94 Jan 1, 2018. Identification of an etiologic pathogen in CSF by mNGS was achieved in
95 101 patients (36.6%). mNGS detected 11 bacterial species, 7 viral species, 2 fungal
96 species, and 2 parasitic species. The five leading positive detections were
97 varicella-zoster virus (17), *Mycobacterium tuberculosis* (14), herpes simplex virus 1
98 (12), Epstein-Barr virus (12), and *Cryptococcus neoformans* (7). False positives
99 occurred in 12 (4.3%) patients with bacterial infections known to be widespread in
100 hospital environments. False negatives occurred in 16 (5.8%) patients and included
101 bacterial, viral and fungal aetiologies.

102 **Conclusions:** mNGS of CSF is a powerful diagnostic method to identify the pathogen
103 for many central nervous system infections.

104

105 **Keywords:** encephalitis, meningitis, metagenomic next-generation sequencing,
106 diagnosis

107 **1. INTRODUCTION**

108 Infectious encephalitis and meningitis are major contributors to the neurological
109 global burden of disease¹⁻⁴. Numerous microorganisms, including bacteria, viruses,
110 fungi, and parasites, can cause encephalitis and meningitis in immunocompetent or
111 immunocompromised hosts; but the clinical manifestations of many infections are
112 non-specific. Using comprehensive conventional diagnostic technologies,
113 microbiological detection of the pathogen is achieved in only 50-80% of cases⁵⁻⁸. The
114 inability to identify the infectious aetiology of encephalitis and meningitis often
115 results in delayed, inadequate, and/or inappropriate treatment.

116 Metagenomic next-generation sequencing (mNGS) is a novel tool that allows for
117 the simultaneous and independent sequencing of thousands to billions of DNA
118 fragments⁹. Cerebrospinal fluid (CSF) is particularly suitable for NGS due to its
119 sterility in healthy individuals. Compared with traditional individual target-specific
120 tests, mNGS can identify pathogens without the input of clinical predictors or prior
121 laboratory results. Several recent studies have demonstrated the capability of mNGS
122 of CSF to identify known and unsuspected pathogens and to discover new
123 microorganisms¹⁰⁻¹⁸. mNGS of CSF is being increasingly utilized in routine clinical
124 settings for the rapid diagnosis of central nervous system infections. However, most
125 published studies are retrospective case reports or case series^{11-17,19-24}, and thus, large
126 prospective studies are needed to demonstrate the clinical impact and
127 cost-effectiveness of mNGS for the diagnosis of meningitis and encephalitis. We
128 undertook a multicentre prospective study to comprehensively evaluate the

129 performance of mNGS of CSF for the diagnosis of central nervous system (CNS)

130 infections compared to conventional microbiological methodologies.

131

132 **2. METHODS**

133 **2.1 Participants and study design**

134 This study was a multicentre prospective cohort assessment of the mNGS of CSF for
135 the diagnosis of suspected infectious encephalitis or meningitis. The participating
136 sites were 20 hospitals located in 10 provinces/municipalities in China. Each hospital
137 is a member of the Beijing Encephalitis Group. Adult patients were eligible for
138 inclusion in the study if they presented with clinical manifestations consistent with
139 either encephalitis or meningitis (Table 1) and if standard diagnostic examinations
140 (Supplementary Table 1) failed to identify an etiological cause within 3 days.

141 Exclusion criteria are shown in Table 1.

142 mNGS were conducted on all CSF specimens. Relevant conventional
143 microbiological studies (e.g. staining, culture, polymerase chain reaction [PCR],
144 serology) were arranged according to the clinical manifestations and the results of
145 mNGS. Conventional microbiological studies were considered the gold standard
146 according to relevant guidelines and/or consensus^{2,25-27} to classify the results of
147 mNGS as true-positive, false-positive, and false-negative. Detected pathogens were
148 classified as etiologic pathogens if the major clinical manifestations of the patient
149 were consistent with that pathogen. All patients were treated based on the results of
150 conventional microbiological testing (or empirically if results were negative)

151 according to the latest clinical guidelines and/or consensus. Patients were followed for
152 at least 30 days to determine the final diagnosis. Demographic data, medical history,
153 laboratory test results (including all conventional microbiological tests),
154 neuroimaging findings, medical therapy, and response to treatment were collected
155 prospectively. Patients enrolled from Jan 1, 2017 to Jan 1, 2018 were included in the
156 final analyses.

157 This study was approved by the institutional review board of Peking Union
158 Medical College Hospital (no. JS-890). Written informed consent was obtained from
159 each patient or their legal surrogate prior to enrolment.

160

161 **2.2 mNGS of CSF**

162 CSF samples were collected according to standard sterile procedures, snap-frozen,
163 and stored at -20°C until they were delivered to the sequencing centre. Because
164 reverse transcription was not performed to prepare DNA libraries, RNA viruses were
165 not investigated in this study. mNGS of the CSF samples was performed using a
166 standard flow that has been successfully used to detect herpes simplex virus 1 (HSV1),
167 HSV2, varicella zoster virus (VZV), *Listeria monocytogenes*, *Brucella*, and *Taenia*
168 *solium*¹²⁻¹⁵.

169 DNA was extracted from 300 μL of CSF and negative ‘no-template’ controls
170 (NTCs). Sequencing was performed on the BGISEQ-100 platform with an average of
171 20 million total reads obtained for each sample. The qualified reads were mapped to
172 the human reference genome using the Burrows–Wheeler Aligner to remove human

173 sequences. The remaining reads were aligned to the database of annotation, which
174 includes the NCBI microbial genome database (<ftp://ftp.ncbi.nlm.nih.gov/genomes/>)
175 to detect pathogens. The sequencing data was analysed in terms of the numbers of raw
176 reads, non-human reads, and reads aligned to the microbial genome database as well
177 as species-specific reads (genus-specific reads for *Mycobacterium tuberculosis* and
178 *Brucella*), reads per million (RPM), and genome coverage (%). The results of mNGS
179 were available in less than 48 hrs.

180

181 **2.3 Criteria for positive results of mNGS of CSF samples**

182 To reduce the influence of potential contamination, we used the following criteria for
183 positive results of CSF mNGS:

184 1) For extracellular bacteria, fungi (excluding *Cryptococcus*), and parasites, the
185 result was considered positive if a species detected by mNGS had a species-specific
186 read number (SSRN) ≥ 30 (RPM ≥ 1.50) that ranked among the top 10 for bacteria,
187 fungi, or parasites. Organisms detected in the NTC or that were present in $\geq 25\%$ of
188 samples from the previous 30 days were excluded but only if the detected SSRN was
189 ≥ 10 -fold than that in the NTC²⁸ or other organisms. Additionally, organisms present
190 in $\geq 75\%$ of samples from the previous 30 days were excluded.

191 2) For intracellular bacteria (excluding *Mycobacterium tuberculosis* and *Brucella*)
192 and *Cryptococcus*, the result was considered positive if a species detected by NGS
193 had a SSRN ≥ 10 (RPM ≥ 0.50)¹³ that ranked among the top 10 for bacteria or fungi.
194 Pathogens detected in the NTC or that were present in $\geq 25\%$ of samples from the

195 previous 30 days were excluded but only if the detected SSRN was ≥ 10 -fold than that
196 in the NTC or other organisms.

197 3) For viruses, *Brucella*, and *Mycobacterium tuberculosis*, the result was
198 considered positive if a species (or genus for *Mycobacterium tuberculosis*
199 [*Mycobacterium tuberculosis* complex, MTC] and *Brucella*) detected by NGS had a
200 SSRN ≥ 3 (RPM ≥ 0.15)^{12,28}. Pathogens detected in the NTC were excluded but only
201 if the detected SSRN was ≥ 10 -fold than that in the NTC. In our previous clinical
202 observations, there were a few cases without *Mycobacterium tuberculosis* infection
203 which contained MTC-specific reads number of 1 in the mNGS results. To mitigate
204 the possibility of false positives, we adopted the criteria of SSRN ≥ 3 rather than
205 SSRN ≥ 1 ²⁴ in this study.

206 The performance of the criteria were evaluated at the finally stage of the study,
207 the original results of mNGS and/or clinical manifestations were used to guide the
208 further testing of conventional microbiological studies.

209

210 **2.4 Statistical analysis**

211 All statistical analyses were conducted using Statistical Package for the Social
212 Sciences (SPSS) version 17.0 and EXCEL 1810. Depending on their distribution, all
213 data are expressed as medians with interquartile ranges (IQRs) or as means \pm standard
214 deviation.

215

216 **3. RESULTS**

217 **3.1 Characteristics of the study participants**

218 287 patients were screened for inclusion in this study (Fig. 1). 11 patients were
219 initially thought to have CNS infections and mNGS was performed. However, these
220 cases were ultimately excluded following the final diagnosis of a non-infectious
221 disease. Of these 11 excluded patients, 10 had negative mNGS results, and 1 patient
222 receiving immunosuppressive therapy was positive for BK polyomavirus. The final
223 cohort included 276 patients in the study. 176 (63.8%) were male and the median age
224 was 42 years (IQR: 26–54 years). The median time from disease-onset to CSF
225 sampling was 10 days (IQR: 5–25 days). The median white blood cell count in CSF
226 was 80/mm³ (IQR: 19–220/mm³). The median CSF monocyte cell count was 36/mm³
227 (IQR: 10–127/mm³). During a follow-up period of 30 days, nine patients died.

228

229 **3.2 Performance of mNGS for diagnosing CNS infections**

230 276 CSF samples were tested by mNGS and conventional microbiological studies. 122
231 samples were positive by mNGS (110 true positive, 12 false positive), 126 were
232 positive by conventional microbiological tests, and 114 total positive results were
233 considered "Etiologic Pathogens" (Table 2). All mNGS results were obtained in less
234 than 48 hours and 101 CSF samples were positive by mNGS before any conventional
235 microbiological tests were positive.

236 Of the patients first diagnosed by mNGS, 16.3% of infections were caused by
237 bacterial, 15.2% by viruses, 2.9% by fungi, and 2.2% by parasites (Fig. 2A).

238 In total, NGS detected 11 bacterial species, of which *M. tuberculosis* (14 cases,

239 13.9%) and *L. monocytogenes* (7.9%) were the most common (Fig. 2B), 7 viral
240 species (BK polyomavirus was not the etiologic pathogen), of which VZV (16.8%)
241 and HSV1 (11.9%) were the most common, 2 fungal species, both of which were
242 *Cryptococcus* (7.9%), and 2 parasitic species, of which *T. solium* (5.0%) was the most
243 common. Nine co-infections with Epstein-Barr virus (EBV) (three with HSV1, two
244 with *Brucella*, one with *Cryptococcus*, one with *S. haemolyticus*, one with *P.*
245 *aeruginosa*, and one with *M. tuberculosis*), two co-infections with cytomegalovirus
246 (CMV) (one with *M. tuberculosis*, and one with *Cryptococcus*), and one co-infection
247 with BK polyomavirus (with HSV1) were detected. The EBV and BK polyomavirus
248 did not appear to be consistent with the clinical manifestations in these two instances
249 of co-infections.

250

251 **3.3 False positive results of CSF mNGS**

252 In the present study, false positives occurred in 12 (4.3%) patients and were primarily
253 associated with bacterial infections (n=12; Table 2), including *E. coli*, *E. faecium*, *A.*
254 *baumannii*, *S. maltophilia*, and *P. aeruginosa*, and a false positive for *Brucella* was
255 also seen. Of note, the false-positive samples contained numerous other bacteria, that
256 could be detected simultaneously by NGS. Using our proposed criteria, there were no
257 false positives for viruses, fungi, or parasites. Although EBV was not the etiologic
258 pathogen in most cases, it was present in the CSF of some patients. Additionally, there
259 was some background contamination in most CSF samples (Supplementary Table 2)
260 but these organisms did not meet the criteria for a positive result.

261

262 **3.4 False negative results of CSF mNGS**

263 In the present study, false negatives occurred in 16 (5.8%) patients (Table 2) and were
264 associated with bacterial, viral and fungal infections. The false negative cases of
265 bacterial infection were all treated with antibiotics prior to sequencing. In the false
266 negative cases of viral infection, 1 or 2 SSRNs were detected in the samples but did
267 not satisfy the proposed criteria for a positive mNGS result. If the criteria for a
268 positive result was relaxed to a SSRN ≥ 1 (RPM ≥ 0.05), there were no false negative
269 cases of HSV1 or VZV or false positive cases of HSV1, HSV2 or VZV. In this study,
270 if we adopted the alternate criteria SSRN ≥ 1 (RPM ≥ 0.05) for viruses and
271 *Mycobacterium tuberculosis*, there would be additional potential false positives,
272 including 30 EBV, 7 CMV and 5 *Mycobacterium tuberculosis* infections. It should be
273 pointed out that the possibility of *Mycobacterium tuberculosis* infection in the 5 cases
274 cannot be ruled out based on the clinical and paraclinical manifestations, because the
275 conventional microbiological methods might fail to detect the *Mycobacterium*
276 *tuberculosis*. Of note, there were three false negative cases of *Cryptococcus* infection.

277

278 **4. DISCUSSION**

279 To the best of our knowledge, the present study is the first to assess the performance
280 of mNGS for pathogen identification in a large prospective cohort of patients with
281 suspected CNS infections. Specifically, our study compared results of mNGS of CSF
282 to conventional microbiological studies and proposed new criteria for validating a

283 mNGS result as positive for therapeutic decision-making. Our results suggest that
284 NGS can provide a quicker and more accurate etiologic pathogen identification than
285 conventional microbiological methods. However, patients in the present study were
286 only eligible to be assessed by mNGS if conventional microbiological studies, e.g.
287 routine bacterial stains and cultures, India ink preparation, targeted PCR tests,
288 serological tests, failed to identify an etiologic cause within 3 days. Thus, the
289 application of CSF mNGS in the clinical setting of this study could be regarded as a
290 quasi-first line method for diagnosing CNS infectious diseases.

291 mNGS is a high-throughput sequencing technique without the requirement of
292 prior information, allowing detection of unsuspected or novel organisms. Importantly,
293 mNGS can detect unsuspected pathogens that clinicians may fail to consider because
294 of atypical clinical manifestations. Many cases of neurological infections have been
295 unexpectedly diagnosed by mNGS of CSF^{11,22,29,30} similar to the present study for the
296 cases of *L. monocytogenes*, *Brucella* and *T. solium*¹²⁻¹⁴. In addition, as demonstrated
297 in previous studies^{10,20,21} and in the present study for the case of encephalitis caused
298 by *Suid herpesvirus 1*³¹, mNGS of CSF has the ability to identify novel aetiologies of
299 CNS infections. Furthermore, NGS can detect unexpected co-infections that may
300 guide appropriate targeted treatment. For example, we detected co-infections of CMV
301 and *Cryptococcus*. In routine clinical practice, if conventional microbiological
302 methods detect *Cryptococcus*, then no further tests for other microorganisms other
303 than HIV are usually performed. Finally, mNGS of CSF may be an appropriate tool
304 for ruling out a broad spectrum of potential CNS infectious diseases prior to

305 concluding a final diagnosis of autoimmune diseases, such as autoantibody-negative
306 autoimmune encephalitis.

307 Contamination of samples during specimen collection and/or processing is a
308 major challenge when interpreting mNGS results. To reduce the potential influence of
309 contamination, we defined strict criteria for positive mNGS results. The various types
310 of contamination observed in the present study could be divided into two groups: (1)
311 microorganisms commonly associated with background contamination that did not
312 meet the criteria for a positive result (Supplementary Table 2) and (2) false positive
313 detections that fulfilled our criteria for a positive mNGS result but were not consistent
314 with the patients' clinical presentation and features. The contaminations derived
315 primarily from the following sources: (1) laboratory practices (Parvovirus NIH CQV
316 is a contaminant from silica column-based nucleic acid extraction kits)³²; (2) reagents
317 (*Bradyrhizobium*, *Burkholderia* and *Ralstonia* are common contaminants used in
318 industrial ultrapure water systems)^{33,34}; (3) environment (*E. coli*, *P. aeruginosa*, *E.*
319 *faecium* and Torque teno virus are widespread pathogens in hospital environment)^{35,36};
320 (4) skin or other body flora (*P. acnes*, *M. globose*, *E. coli* and *S. epidermidis* are
321 widely associated with the human skin flora)^{37,38}. False positive results are very likely
322 to misguide treatment, and therefore, clinicians should be cautious when interpreting
323 positive mNGS detection of extracellular bacteria or fungi that are widespread in
324 hospital environments, especially when many species of bacteria are detected in a
325 single NGS test. On the other hand, positive mNGS detection of viruses and parasites
326 are not likely to be false positives.

327 Negative mNGS results do not necessarily exclude an infectious cause for the
328 patient's illness. In the present study, false negative mNGS results occurred in 5.8% of
329 cases and included bacterial, viral, and fungal infections. The prior use of antibiotics
330 can affect the detection rate of bacteria. Low SSRN values (1 or 2 reads) can be seen
331 in false negative results for virus detection indicating that other microbiological tests
332 should be conducted to confirm a diagnosis when the SSRN is low for viruses. False
333 negative cases have been reported for *Cryptococcus* due to fungal counts below the
334 lower limit of detection for nucleic acid amplification tests^{39,40}. The criteria of SSRN
335 ≥ 3 rather than $\text{SSRN} \geq 1$ might introduce false-negative cases for *Mycobacterium*
336 *tuberculosis* infections. As a screening method, the pathogens detected by mNGS
337 might provide clinical clues for further investigation even if the results do not fulfil
338 the specified criteria for a positive result.

339 Our results indicate that mNGS of CSF is a very useful test for the diagnostic
340 evaluation of patients with suspected CNS infections. mNGS has already become a
341 first-line laboratory method in the response to emerging infectious diseases and
342 outbreaks of infectious diseases^{41,42}. Moreover, increasing evidence provides a
343 rationale for using mNGS as a first-line diagnostic test for chronic and recurring
344 encephalitis and as a second-line test for acute encephalitis²³. In the present study,
345 more than one-third of patients were first diagnosed by mNGS of CSF within 48 hrs,
346 indicating that this technique can be extremely useful for rapid clinical
347 decision-making. mNGS of CSF should be considered as a first-line test for acute
348 CNS infections when (1) a patient is critically ill and requires prompt and precise

349 therapy, (2) the clinical manifestations are non-specific and numerous target-specific
350 tests are simultaneously applied to identify an infectious cause, 3) a broad spectrum of
351 potential pathogens needs to be ruled out to diagnose a suspected autoimmune
352 encephalitis, and (4) rare or novel pathogens are suspected for which standard
353 target-specific tests are not available.

354 There are several limitations regarding the current use of mNGS of CSF in
355 routine clinical settings. First, mNGS is not available in many hospitals and the cost is
356 usually much higher than target-specific tests. Second, the detection of DNA of a
357 certain pathogen does not necessarily prove that it is responsible for the patient's
358 clinical presentation and features^{23,27}. In the present study, some patients were positive
359 for EBV but had other more likely aetiologies of their infections. EBV DNA has often
360 been identified together with other microorganisms in CSF⁴³. Finally, mNGS cannot
361 detect microorganisms that are not included in microbial genome databases.

362 Our study has several limitations. Reverse transcription was not performed to
363 construct a DNA library and therefore, RNA viruses could not be detected by mNGS.
364 Next-generation RNA sequencing should be performed in future prospective studies.
365 The number of CNS infections caused by each pathogen were not sufficient to assess
366 the sensitivity and specificity of mNGS for individual pathogens. Finally, we may
367 have underestimated the sensitivity of mNGS of CSF for diagnosing CNS infections
368 by employing strict criteria for a positive mNGS result and using conventional
369 microbiological methods as the gold standard.

370

371 **List of abbreviations**

372 mNGS: metagenomic next-generation sequencing

373 CSF: cerebrospinal fluid

374 CNS: central nervous system

375 PCR: polymerase chain reaction

376 HSV: herpes simplex virus

377 VZV: varicella zoster virus

378 NTC: 'no-template' controls

379 RPM: reads per million

380 EBV: Epstein-Barr virus

381 CMV: cytomegalovirus

382 SSRN: species-specific read number

383

384 **Declarations**

385 *Ethics approval and consent to participate*

386 This study was approved by the institutional review board of Peking Union Medical

387 College Hospital (no. JS-890). Written informed consent was obtained from each

388 patient or their legal surrogate prior to enrolment.

389 *Consent for publication*

390 Not applicable.

391 *Availability of data and materials*

392 The datasets used and/or analysed during the current study are available from the

393 corresponding author on reasonable request.

394 ***Competing interests***

395 Author Honglong Wu was employed by company BGI-Tianjin and BGI-Shenzhen.

396 Author Yongjun Li was employed by company BGI-Shenzhen. All other authors

397 declare no competing interests.

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402 ***Author's contributions***

403 HG and SF contributed to the study conception design. All the authors participated in

404 the discussion of the study design. SF, XW, YF, JS, YZ, WZ, XQ, CW, LL, LQ, SW,

405 HJ, HL, WY, WZ, XL, SL, YW, YP, HR, YZ, JW, BP, LC, WL, and HG enrolled

406 patients and collected the clinical data. HW, YL and WC performed NGS,

407 bioinformatics analysis, and PCR. SF, HG, XW, and WL analyzed the data. SF wrote

408 the first draft of the manuscript after discussions with HG, XW and WL. HW wrote

409 portions of the Methods section. HG, JHC, TG and WC contributed to manuscript

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413

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569 **Table 1. Case definitions and exclusion criteria for encephalitis and meningitis**

Case definitions for encephalitis
Major criteria (required)
Patients presenting to medical attention with altered mental status (decreased or altered level of consciousness or personality change) lasting ≥ 24 hrs with no alternative cause identified, and/or generalized or partial seizures not fully attributable to a pre-existing seizure disorder or a simple febrile seizure.
Minor criteria (≥ 2 points)
Documented fever $\geq 38^{\circ}\text{C}$ within the 72 hrs before or after presentation
New onset of focal neurologic findings
CSF WBC count $\geq 5/\text{cubic mm}$
Abnormality of brain parenchyma on neuroimaging suggestive of encephalitis that is either new from prior studies or appears acute in onset
Abnormality on EEG that is consistent with encephalitis and not attributable to another cause.
Exclusion criteria for encephalitis
≤ 28 days of age
Non-infectious encephalitis, such as autoimmune disorders, paraneoplastic syndromes, NMOSD, neuropsychiatric involvement of rheumatic diseases
HIV or syphilis infection
History of recent (within 4 weeks before the onset of disease) vaccination
Meningitis without clinical brain parenchyma involvement
Absolute contraindications for lumbar puncture;
Traumatic LP with obvious blood-contaminated CSF
Pregnancy
Refusal to sign the informed consent
Case definitions for meningitis
Patients presenting to medical attention with at least two of the four symptoms of headache, fever (documented fever $\geq 38^{\circ}\text{C}$ within the 72 hrs before or after presentation), neck stiffness, decreased level of consciousness (defined by a Glasgow Coma Scale score below 14)
CSF white blood cell count $\geq 5/\text{cubic mm}$
Exclusion criteria for meningitis
≤ 28 days of age
HIV or syphilis infection
Meningeal malignancy confirmed by CSF cytology
Traumatic LP with obvious blood-contaminated CSF
Pregnancy
Refusal to sign the informed consent

570 **Table 2.** Performance of mNGS of CSF compared to conventional microbiological
 571 studies (gold standard) for the diagnosis of CNS infections

	TP*	FP	FN	Etiologic Pathogen
Bacteria				
<i>Mycobacterium tuberculosis</i>	14	0	1	15
<i>Listeria monocytogenes</i>	8	0	0	8
<i>Brucella</i>	7	1	1	8
<i>Streptococcus pneumoniae</i>	5	0	0	5
<i>Klebsiella pneumoniae</i>	3	0	0	3
<i>Streptococcus intermedius</i>	2	0	0	2
<i>Haemophilus influenzae</i>	2	0	0	2
<i>Vibrio vulnificus</i>	1	0	0	1
<i>Staphylococcus hominis</i>	1	0	0	1
<i>Escherichia coli</i>	0	2	0	0
<i>Enterococcus faecium</i>	0	2	0	0
<i>Acinetobacter baumannii</i>	1	2	0	1
<i>Stenotrophomonas maltophilia</i>	1	1	0	1
<i>Pseudomonas aeruginosa</i>	0	4	1	1
<i>Staphylococcus aureus</i>	0	0	1	1
<i>Staphylococcus haemolyticus</i>	0	0	1	1
DNA Viruses				
Varicella-zoster virus	17	0	4	21
Herpes simplex virus 1	12	0	1	13
Epstein-Barr virus	12	0	3	6
Cytomegalovirus	4	0	0	2
Herpes simplex virus 2	2	0	0	2
<i>Suid herpesvirus 1</i>	2	0	0	2
BK polyomavirus	1	0	0	0
John Cunningham virus	1	0	0	1
Fungi				
<i>Cryptococcus neoformans</i>	7	0	3	10
<i>Cryptococcus gattii</i>	1	0	0	1
Parasites				
<i>Taenia solium</i>	5	0	0	5
<i>Angiostrongylus cantonensis</i>	1	0	0	1
Totals	110	12	16	114

572 * TP: true-positive; FP: false-positive; FN: false-negative; mNGS: metagenomic

573 next-generation sequencing; CSF: cerebrospinal fluid; CNS: central nervous system

574 **Figure legends**

575 **Figure 1.** Flowchart of patient enrolment and exclusion.

576

577 **Figure 2.** Distribution of causative pathogens in patients with suspected CNS

578 infections initially detected by NGS of CSF. (A) Of the 36.6% patients first diagnosed

579 with NGS of CSF, 16.3% were diagnosed with bacterial infections, 15.2% with viral

580 infections, 2.9% with fungal infections, and 2.2% with parasitic infections. (B) NGS

581 detected 11 bacterial species, the most common of which were *M. tuberculosis*

582 (13.9%) and *L. monocytogenes* (7.9%), 7 viral species, the most common of which

583 were VZV (16.8%) and HSV1 (11.9%), 2 fungal species, both of which were

584 *Cryptococcus* (7.9%) species, and 2 parasitic species, the most common of which was

585 *T. solium* (5.0%).

287 patients screened and sequenced

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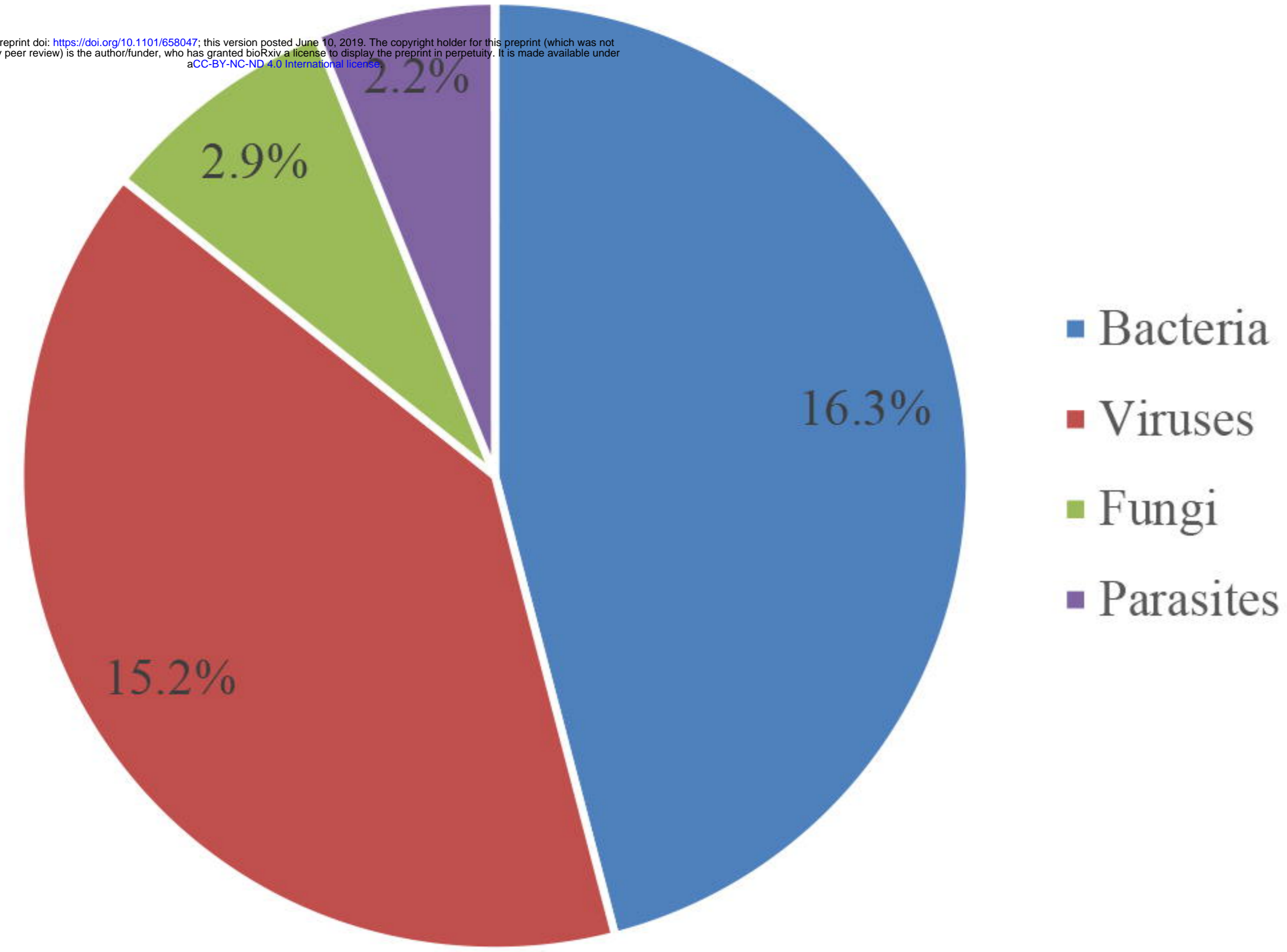
11 patients excluded:

- Systematic vasculitis: 2 patients
- Rasmussen encephalitis: 1 patient
- Neuromyelitis optica spectrum disorders: 1 patient
- Idiopathic hypertrophic pachymeningitis: 1 patient
- Sjogren's syndrome: 1 patient
- Primary central nervous system lymphoma: 1 patient
- Autoinflammatory diseases: 1 patient
- Primary angiitis of the central nervous system: 1 patient
- Glioma: 1 patient
- Neuro-Behcets's syndrome: 1 patient

276 patients included

A

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**B**