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THÈSE DE DOCTORAT

Soutenue à Aix-Marseille Université
Le 30 juin 2022 par :

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Diagnostic moléculaire des méningites communautaires : Approche basée sur le séquençage direct par métagénomique

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Microbes Evolution Phylogeny and Infections MEPHI- Aix Marseille Université, Institut de Recherche pour le Développement IRD, IHU Méditerranée Infection, Marseille, France.

VBIC, INSERM U1047, Université de Montpellier, service de Microbiologie et Hygiène Hospitalière, CHU Nîmes, Nîmes, France.

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Affidavit

Je soussigné, Madjid MORSLI, déclare par la présente que le travail présenté dans ce manuscrit est mon propre travail, réalisé sous la direction scientifique du Professeur Michel DRANCOURT à Marseille et la codirection du Professeur Jean Philippe LAVIGNE à Nîmes, dans le respect des principes d'honnêteté, d'intégrité et de responsabilité inhérents à la mission de recherche. Les travaux de recherche et la rédaction de ce manuscrit ont été réalisés dans le respect à la fois de la charte nationale de déontologie des métiers de la recherche et de la charte d'Aix-Marseille Université relative à la lutte contre le plagiat.

Ce travail n'a pas été précédemment soumis en France ou à l'étranger dans une version identique ou similaire à un organisme examinateur.

Fait à Marseille, le 30 avril 2022.

Affidavit

I, undersigned, Madjid MORSLI, hereby declare that the work presented in this manuscript is my own work, carried out under the scientific direction of Professor Michel DRANCOURT and codirection of Professor Jean Philippe LAVIGNE, in accordance with the principles of honesty, integrity and responsibility inherent to the research mission. The research work and the writing of this manuscript have been carried out in compliance with both the French national charter for Research Integrity and the Aix-Marseille University charter on the fight against plagiarism.

This work has not been submitted previously either in this country or in another country in the same or in a similar version to any other examination body.

Place Marseille, April/30th, 2022.

Liste de publications et participation aux conférences

1) Liste des publications réalisées dans le cadre du projet de Thèse :

1. Morsli M, Lavigne JP, Drancourt M. Direct metagenomic diagnosis of community-acquired meningitis: state of the art. a review. Accépté (Frontiers in Microbiology).
2. Morsli M, Salipante F, Kerharo Q, Boudet A, Stephan R, Dunyach-Remy C, Zandotti C, Lavigne JP, Drancourt M. Warming-driven outbreaks of community-acquired meningitis syndrome in southern France (Soumis à Medicine).
3. Morsli M, Vincent JJ, Milliere L, Colson P, Drancourt M. Direct next-generation sequencing diagnosis of echovirus 9 meningitis, France. Eur J Clin Microbiol Infect Dis. 2021; 40:2037–9.
4. Morsli, M.; Zandotti, C.; Morand, A.; Colson, P.; Drancourt, M. Direct Diagnosis of Echovirus 12 Meningitis Using Metagenomic Next Generation Sequencing. Pathogens 2021, 10, 610.
5. Morsli M, Bechah Y, Coulibaly O, Toro A, Fournier P, Houhamdi L, et al. Direct diagnosis of *Pasteurella multocida* meningitis using next-generation sequencing. The Lancet Microbe. 2022;3:e6.
6. Morsli M, Kerharo Q, Delerce J, Roche P, Troude L, Drancourt M. Haemophilus influenzae Meningitis Direct Diagnosis by Metagenomic Next-Generation Sequencing: A Case Report. Pathogens. 2021; 10, 461.
7. Morsli M, Kerharo Q, Amrane S, Parola P, Fournier PE, Drancourt M. Real-time whole genome sequencing direct diagnosis of *Streptococcus pneumoniae* meningitis: a case report. J Infect. 2021; 10:14–6.
8. Morsli M, Boudet A, Kerharo Q, Stéphan R, Salipante F, Dunyach-Remy C, Houhamdi L, Fournier PE, Lavigne JP, Drancourt M. Real-time metagenomics-based diagnosis of community-acquired meningitis: a prospective series, southern France. (eBioMedecine, en révision).
9. Morsli, M., Anani, H., Bréchard, L., Delerce, J., Bedotto, M., Fournier, P. E., & Drancourt, M. LamPORE SARS-CoV-2 diagnosis and genotyping: A preliminary report. J Clin Virol 2021; 138: 1–2.
10. Morsli M, Faltot M, Astier H, Le Dault E, Chaudier B, Garnotel E, Baron SA, Drancourt M, Real-time next-generation sequencing on shell-vial culture to contribute to diagnosis of lymphatic tuberculosis: a case report, Diagnostic Microbiology and Infectious Disease,Volume 101, Issue 3,2021, 115492.
11. Robinne S, Saad J, Morsli M, Hamidou ZH, Tazerart F, Drancourt M and Baron SA. Rapid Identification of *Mycobacterium tuberculosis* Complex Using Mass Spectrometry: A Proof of Concept. Front. Microbiol. 2022;

13:753969. doi: 10.3389/fmicb.2022.753969

12. Harouna Hamidou Z, Morsli M, Mamadou S, Drancourt M, Saad J (2022) Emergence of multi-drug-resistant Mycobacterium tuberculosis in Niger: A snapshot based on whole-genome sequencing. PLoS Negl Trop Dis 16(5): e0010443.
13. Fayolle M, Morsli M, Gelis A, Chateauraynaud M, Yahiaoui-Martinez A, Sotto A, Lavigne JP, Dunyach-Remy C. The Persistence of *Staphylococcus aureus* in Pressure Ulcers: A Colonising Role. Genes 2021, 12, 1883.
14. Guindo CO, Morsli M, Bellali S, Drancourt M, Grine G, A *Tetragenococcus halophilus* human gut isolate. Current Research in Microbial Sciences, Volume 3, 2022, 100112, ISSN 2666-5174.

2) Participation aux conférences et écoles d'été au cours de la période de Thèse :

1. Le 29/01/2021: Vision conference: American Society of Microbiology. Diagnostic par métagénomique des infections du système nerveux central.
2- Le 18/11/2021 Journées utilisateurs iSeq™ 100
3- Le 30/11/2021 Nanopore Community Meeting Online 2021
4- Formateur en NGS et métagénomique en temps réel dans le cadre de projet MICAFRICA ; Projet Européen coordonné par le Prof. L KESKES, Université de Sfax, associant quatre établissements de recherche (Tunisie, Italie, France).
5- Participation aux work in progress de chaque mardi à l'IHU Méditerranée Infection
6- Participation aux Journées InfectioPole Sud, Marseille : en 2020 et 2021.
7- London Calling 2022; par Vision "Diagnosis of life-threatening infections by direct real-time metagenomic".

Avant-propos

Le format de présentation de cette Thèse correspond à une recommandation de la spécialité Maladies Infectieuses et Microbiologie, à l'intérieur du Master des Sciences de la Vie et de la Santé, qui dépend de l'Ecole Doctorale des Sciences de la Vie de Marseille. Le candidat est amené à respecter des règles qui lui sont imposées et qui comportent un format de Thèse utilisé dans le Nord de l'Europe et qui permet un meilleur rangement que les Thèses traditionnelles. Par ailleurs, la partie introduction et bibliographie est remplacée par une revue envoyée dans un journal afin de permettre une évaluation extérieure de la qualité de la revue et de permettre à l'étudiant de commencer le plus tôt possible une bibliographie exhaustive sur le domaine de cette Thèse.

En outre, la thèse est présentée sur article publié, accepté ou soumis associé d'un bref commentaire donnant le sens général du travail. Cette forme de présentation a paru plus en adéquation avec les exigences de la compétition internationale et permet de se concentrer sur des travaux qui bénéficieront d'une diffusion internationale

Professeur Didier RAOULT.

Résumé

Les méningites communautaires sont des urgences vitales, dont le pronostic est partiellement associé à l'agent microbien pathogène et son génotype. Plus de 100 micro-organismes différents ont été impliqués dans les infections de système nerveux central après leur détection et leur identification dans le liquide céphalo-rachidien (LCR), à travers le monde. La culture de LCR a longtemps été le gold standard du diagnostic des infections de système nerveux central mais actuellement, ce diagnostic de routine microbiologique est basé sur la détection par PCR multiplexe en temps réel des pathogènes les plus fréquents. Cependant, le génotypage des microbes responsables tel que les Entérovirus qui couvrent plus de 300 sérotypes différents, dont 110 infectent les patients, ainsi que le génotypage et l'antibiogramme des bactéries pathogènes, nécessitent des investigations *in-vitro* supplémentaires. Notre étude rétrospective de 20,779 LCR prélevés dans le cadre du diagnostic des méningites communautaires au cours de 61 mois, analysés dans les laboratoires de microbiologie clinique de Nîmes et Marseille a montré l'absence de documentation dans plus de 89% des cas. La métagénomique NGS est un outil potentiel pour le diagnostic direct des méningites infectieuses à partir de LCR en détectant le génome pathogène sans PCR préalable. Dans ce travail de Thèse, nous avons répondu à quatre problématiques : 1) Mise à jour du répertoire des agents pathogènes causatifs de méningites, détectés par métagénomique NGS directe du LCR. 2) Epidémiologie des méningites communautaires à Nîmes et Marseille. 3) Amélioration de diagnostic et génotypage des méningites à Entérovirus. 4) Développement et implantation d'un protocole "one-shot" utilisant la métagénomique en temps réel pour le diagnostic, le génotypage et l'antibiogramme *in-silico* des méningites au laboratoire point-de-soins (POC).

Mots-clés : Méningites communautaires, liquide céphalorachidien, infection, épidémie, méningites non-documentées, étiologie, génome pathogène, métagénomique, résistance, génotypage, séquençage en temps réel, NGS, diagnostic, point-de soins.

Abstract

Community-acquired meningitis is a life-threatening condition, whose prognosis is partially dependent on the causative pathogen and its genotype. More than 100 different microorganisms have been involved in central nervous system infections after their detection and identification in cerebrospinal fluid (CSF), worldwide. The CSF culture was for a long time the gold standard for the diagnosis of central nervous system infections but nowadays, this routine microbiological diagnosis is based on the detection by multiplexed RT-PCR of the most frequent pathogens. However, the genotyping of causative pathogens such as Enteroviruses which cover more than 300 different serotypes, of which 110 infect humans, as well as the genotyping and antibiogram of pathogenic bacteria, require additional *in-vitro* investigations. Our retrospective study of 20,779 CSFs collected for the diagnosis of community-acquired meningitis during 61 months, analyzed in the clinical microbiology laboratories of Nîmes and Marseille showed the absence of documentation in more than 89% of cases. Metagenomics NGS is a potential tool for the direct diagnosis of infectious meningitis from CSF samples by detecting the pathogenic genome without prior PCR. In this thesis work, we have addressed four issues: 1) Update of the repertoire of meningitis causative pathogens detected by direct metagenomic NGS of CSF. 2) Epidemiology of community-acquired meningitis in Nîmes and Marseille. 3) Improvement of the diagnostic and genotyping of Enterovirus meningitis. 4) Development and implementation of a "one-shot" protocol using real-time metagenomics for diagnosis, genotyping, and *in-silico* antibiotic susceptibility testing of community-acquired meningitis in the point-of-care (POC) laboratory.

Keywords: Community-acquired meningitis, cerebrospinal fluid, infection, outbreak, undocumented meningitis, etiology, pathogen genome, metagenomics, resistance, genotyping, real-time sequencing, NGS, diagnosis, point-of-care.

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Durant ces trois années de thèse, de nombreuses personnes ont croisé mon chemin et ont participé à un moment ou un autre à l'aboutissement de mon Doctorat.

Je tiens tout d'abord à remercier le Professeur Didier RAOULT de m'avoir accepté dans son institut pour réaliser ma thèse de doctorat. Je tiens à remercier infiniment le Professeur Michel DRANCOURT et le Professeur Jean Philippe LAVIGNE de m'avoir accepté dans leurs équipes pour réaliser mon projet de thèse et pour leur qualité d'encadrement et leurs soutiens.

Je tiens à remercier la fondation FMI d'avoir financé ma thèse. Je tiens à remercier tous mes collègues et collaborateurs pour les moments qu'on a partagés. Je remercié mes amis Mahmoud, Hacène, Handi, Karim, Ferhat, Wafa, Yasmine, Cheick, Abdou, Djamel, Younes, Ihab, Rym, Hamadou et toute la Drancourt team sans oublier toutes les personnes que j'ai rencontré à Marseille et à Nîmes. Je remercié aussi mes anciens collègues et amis spécialement Marie et sa petit Famille, Hamid, Nabil, Mahdi, Ali, Rachid, Yacine et toute personne ayant participée d'une façon directe ou indirecte dans ce travail.

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Liste des abréviations

ADN : Acide désoxyribonucleique
AOR : Adjusted odd ration
APHM : Assistance publique des hopitaux de Marseille
ARN : Acide ribonucléique
CAM : Community-acquired meningitis
CHU : Centre hospitalo-universitaire
CNS : Central nervous system
CSF : Cerebrospinal fluid
DNA CS : DNA controls
DNA : Desoxy-ribinucleic acid
HHV : Human herpes virus
HSV : Herpes simplex virus
IHU: Institut hospitalo-universitaire
INSIRM : Institut national de la santé et de la rechreche médicale
IRD : Institut de recherche et de développement
LAMP : Loop-mediated isothermal amplification
LCR : Liquide cephalorachidien
LFB : Long fragment Buffer
MCA : Multiple correspondance analysis
ME : Méningite/encéphalie
MEPHI : Microbes, Evolution, Phylogénie, et Infection
MLST : Multi-locus sequence typing
mNGS: Metagenomic next generation sequencing
NCBI: National center of bioinformatic
NGS : Next generation sequencing
PCR : Polymerase chain reaction
POC : point-of-care
PRISMA: Preferred Reporting Items for Systematic Reviews and Meta-Analyses
RTM: Real-time metagenomics
RNA : Ribonucleic acid
RT-PCR : Real-time plymerase chain reaction
RT-LAMP : Reverse-transcription loop-mediated isothermal amplification
SD : Standatd diviation
VIBIC : Virulence bactérienne et infections chroniques
WGS: Whole genome sequencing
WNV : West Nile virus
VZV : Variclla zoster virus

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Introduction

Le terme de méningite désigne une inflammation des trois enveloppes qui recouvrent le cerveau et la moelle épinière (1,2). Dans ce travail de Thèse, nous avons travaillé sur les méningites infectieuses communautaires, qui causent plus de 50% des cas de méningites, souvent consécutives à des infections virales, plus rarement bactériennes, exceptionnellement fongiques et parasitaires (2,3). Chaque année, plus de 1.3 millions de cas de méningites infectieuses sont rapportés à travers le monde, dont plus de 40% de cas mortels essentiellement en absence de traitement (4). Les méningites virales sont les plus fréquentes causées d'une part par les *Herpesviridae* incluant l'*Herpès Simplex Virus* 1 et 2 et *Varicelle zoster virus* en majorité chez les personnes âgées (4,5). En Europe, les Entérovirus sont les étiologies les plus fréquemment documentées de méningites communautaires infectant beaucoup plus les populations jeunes [6–8], dont le pronostic est associé au sérotype l'Entérovirus (8–12). Les épidémies d'Entérovirus sont enregistrées d'une façon annuelle durant la saison estivale (6,8,13). Les méningites bactériennes sont les plus sévères pouvant progresser vers le décès en moins de 48 heures en absence de traitement (14) causant plus de 50% des cas de mortalité des méningites infectieuses (14–16); dans la plupart des cas sont associées à des infections par *Streptococcus pneumoniae, Neisseria meningitidis, Haemophilus influenzae* et *Streptococcus agalactiae* et plus rarement *Listeria monocytogenes* et d'autres espèces bactériennes (14–19). Les méningites fongiques souvent représentées par des infections à *Cryptococcus neoformans/gattii,* détectés chez des personnes immunodéprimées avec une incidence très variable selon les pays alors que les méningites parasitaires sont presque inexistantes en Europe (20–23). La culture du liquide céphalorachidien (LCR) reste le gold standard pour le diagnostic des méningites infectieuses en routine (15,24). Cependant en pratique de routine, le diagnostic moléculaire d'urgence des méningites communautaires est basé sur la détection multiplexée de séquences nucléotidiques des pathogènes les plus fréquemment identifiés dans le LCR par amplification en temps réel par PCR multiplex, pour laquelle plusieurs trousses diagnostiques sont commercialisées (1,25–31). La caractérisation de pathogène, le génotypage et la détermination du profil de sensibilité aux antibiotiques pour les bactéries, nécessitent des investigations supplémentaires *in-vitro*; parfois limitées par la non-disponibilité d'un système PCR spécifique et l'absence de culture des bactéries, singulièrement lorsque le LCR a été prélevé après le début d'un traitement antibiotique empirique efficace (28).

Pour pallier ces limites, nous avons développé des protocoles expérimentaux permettant l'implantation de la métagénomique par séquençage à haut débit (mNGS) directe du LCR, pour permettre la détection du génome pathogène dans le LCR comme le font les systèmes multiplexés de routine (11,32–35), en apportant des informations supplémentaires concernant le génotypage et l'antibiogramme *in-silico* en une seule étape, contribuant à la détection de nouveaux variants pathogènes (11,36–38) et pathogènes qui ne sont pas détectés en routine (39–42). Au cours de cette Thèse nous avons implémenté le séquençage métagénomique en temps réel au laboratoire point-de-

soins (POC) pour le diagnostic “one-shot “ comportant détection et identification, génotypage et profile de sensibilité dans un temps réduit qui ne dépasse pas une demi-journée directement à partir de LCR (37,42,43).

Pour débuter nos travaux de Thèse, nous avons réalisé une revue exhaustive de la littérature sur l’utilisation de la métagénomique dans le diagnostic des méningites communautaires uniquement à partir de LCR. Ce travail a mis à jour nos connaissances sur les études réalisées sur ce sujet durant ces dernières années (2015-2021).

Dans un second temps, nous avons mené une étude rétrospective sur 61 mois sur le diagnostic et les épidémies de méningites communautaires à Marseille et à Nîmes dans le but de mettre à jour le répertoire des infections de système nerveux central dans cette région du Sud de la France. Puis nous sommes intéressés à l’amélioration de diagnostic et le génotypage des méningites à Entérovirus par métagénomique NGS, suivant deux approches : par enrichissement dans un premier temps puis par déplétion de l’ADN humain dans le LCR, ce qui a mené à identifier pour la première fois le génotype Echovirus 12 dans un LCR d’un bébé de moins de six mois. Nous avons ensuite développé un protocole de métagénomique et d’identification en temps réel des microorganismes à partir de LCR, et nous avons implémenté une plateforme de séquençage en temps réel en utilisant Oxford Nanopore MinION pour identification rapide des agents pathogènes directement à partir de LCR dans un workflow total qui ne dépasse pas quatre heures suivant un pipeline maison.

Chapitre I: Revue de literature: Direct Metagenomic Diagnosis of Community-Acquired Meningitis: State of the Art.

Préambule

Dans cette revue exhaustive de la littérature publiée entre 2015 et 2021, nous avons actualisé nos connaissances sur l'usage de la méthode mNGS pour le diagnostic des méningites communautaires. En utilisant la méthode bibliographique PRISMA (44), nous avons retenu 50 articles éligibles, comportant 25/50 articles publiés par des équipes Chinoises, les autres articles étant publiés par des équipes Américaines et Françaises (incluant nos travaux de Thèse). En particulier, notre revue incluant 1.228 patients avec un syndrome méningé communautaire indique un total de 116 pathogènes identifiés dans les LCR des patients diagnostiqués par métagénomique et par les méthodes classiques en routine, dont plus de 50% ont été identifiés uniquement par mNGS. Notre revue nous a indiqué que, par comparaison à l'isolement des pathogènes par culture et leur détection par immunodétection des antigènes dans le LCR, l'approche mNGS réduit le temps de travail en une seule étape permettant de diagnostiquer, génotyper et investiguer le profil de sensibilité et de résistance aux antibiotiques directement à partir de LCR. Également par comparaison à l'approche PCR multiplexe couramment utilisée au laboratoire POC, qui détecte un nombre limité de pathogènes ne couvrant pas certaines situations médicales spécifiques telles que les méningites communautaires du patient immunodéprimé, l'approche mNGS offre la possibilité de détecter et d'identifier des pathogènes peu ou pas du tout diagnostiqués en routine y compris en cas de très faible charge pathogène dans le LCR (41,42,45–47). Basé sur cette revue, nous avons travaillé à la mise au point de protocoles pour la mise en place de l'approche mNGS et leur évaluation, dans deux laboratoires de Microbiologie Médicale dans lesquels nous avons réalisé nos travaux de Thèse au CHU de Nîmes (Prof. JP LAVIGNE) et au CHU de Marseille (Prof. M. DRANCOURT) dans la perspective de leur utilisation en routine, en complément de l'approche PCR multiplexée.

Article 1

Review: Direct Metagenomic Diagnosis of Community-Acquired Meningitis: State of the Art.

Madjid MORSLI [1, 2], Jean Philippe LAVIGNE [3], Michel DRANCOURT [1, 2, 4,]

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Direct metagenomic diagnosis of community-acquired meningitis: state of the art.

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The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest

Author contribution statement

MM: Literature search, data collection, data cleaning, data interpreting, validation and writing of the manuscript. LJP and DM, design of the study, data interpreting, validation, funding, critically reviewing the manuscript, coordination, and direction of the work. All authors declare that they have read and approved the manuscript

Keywords

metagenomic next generation sequencing, diagnosis, point-of-care laboratory, Multiplex RT-PCR, culture, pathogen, Genome, antibiotic resistance, genotyping.

Abstract

Word count: 197

Current routine diagnosis of community-acquired meningitis (CAM) by multiplex real-time polymerase chain reaction (RT-PCR) is limited in the number of tested pathogens and their full characterisation, requiring additional in-vitro investigations to disclose genotype and antimicrobial susceptibility. We reviewed 51 studies published through December 2021 reporting metagenomic next generation sequencing (mNGS) directly applied to the cerebrospinal fluid (CSF). This approach, potentially circumventing the above-mentioned limitations, indicated 1,248 investigated patients, and 617 patients dually investigated by routine diagnosis and mNGS, in whom 116 microbes were detected, including 50 by mNGS only, nine by routine methods only, and 57 by both routine methods and mNGS. Of 217 discordant CSF findings, 103 CSF samples were documented by mNGS only, 87 CSF samples by routine methods only, and 27 CSF samples in which the pathogen identified by mNGS was different than that found using routine methods. Overall, mNGS allowed for diagnosis and genomic surveillance of CAM causative pathogens in real-time, with a cost which is competitive with current routine multiplex RT-PCR. mNGS could be implemented at point-of-care laboratories as a part of routine investigations to improve the diagnosis and molecular epidemiology of CAM, particularly in the event of failure of routine assays.

Contribution to the field

Dear Editor, Please find enclosed a manuscript entitled: “Direct metagenomic diagnosis of community-acquired meningitis: state of the art” by Madjid MORSLI and collaborators, that we submit for publication in the “Review Article” category of Frontiers in Microbiology. Current diagnosis of community-acquired meningitis mainly based on multiplex RT-PCR targeting a limited number of pathogens and their full characterization, requiring additional in-vitro investigations to disclose genotype and antimicrobial susceptibility. Here, we reviewed 50 studies published until December 2021 reporting metagenomic next generation sequencing (mNGS) directly applied to the cerebrospinal fluid. Total of 116 pathogens were reported in which 106 (91.4%) including 50 pathogens were identified only by metagenomics. Direct diagnosis of community-acquired meningitis by metagenomic allowed to diagnose routinely and non-routinely diagnosed pathogens. As an advantage mNGS added additional information about genotyping and antibiotic susceptibility by analyzing of the NGS data. The authors think that Frontiers in Microbiology, own to its broad, multidisciplinary, high-level audience, is the appropriate tribune to publish this original contribution, to stimulate similar and complementary studies. All the authors approved this manuscript, which is not under consideration elsewhere. Sincerely, Prof. Michel DRANCOURT, MD, PhD. Corresponding author.

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Direct metagenomic diagnosis of community-acquired meningitis: state of the art.

Madjid Morsli [1, 2], Jean Philippe Lavigne [3] and Michel Drancourt [1, 2, 4,]

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Keywords: metagenomic next generation sequencing, diagnosis, point-of-care laboratory, multiplex RT-PCR, culture, pathogen, genome, antibiotic resistance, genotyping.

ABSTRACT

Current routine diagnosis of community-acquired meningitis (CAM) by multiplex real-time polymerase chain reaction (RT-PCR) is limited in the number of tested pathogens and their full characterisation, requiring additional *in-vitro* investigations to disclose genotype and antimicrobial susceptibility. We reviewed 51 studies published through December 2021 reporting metagenomic next generation sequencing (mNGS) directly applied to the cerebrospinal fluid (CSF). This approach, potentially circumventing the above-mentioned limitations, indicated 1,248 investigated patients, and 617 patients dually investigated by routine diagnosis and mNGS, in whom 116 microbes were detected, including 50 by mNGS only, nine by routine methods only, and 57 by both routine methods and mNGS. Of 217 discordant CSF findings, 103 CSF samples were documented by mNGS only, 87 CSF samples by routine methods only, and 27 CSF samples in which the pathogen identified by mNGS was different than that found using routine methods. Overall, mNGS allowed for diagnosis and genomic surveillance of CAM causative pathogens in real-time, with a cost which is competitive with current routine multiplex RT-PCR. mNGS could be implemented at point-of-care laboratories as a part of routine investigations to improve the diagnosis and molecular epidemiology of CAM, particularly in the event of failure of routine assays.

INTRODUCTION

Community-acquired meningitis (CAM), a life-threatening condition with 40% mortality (Beaman, 2018), warrants a rapid diagnosis, possibly within two hours at a point-of-care (POC) laboratory, providing identification of the causative pathogen among a repertoire of fewer than 20 pathogens (Launes *et al.*, 2017; Leber *et al.*, 2016; Soucek *et al.*, 2019; Vincent *et al.*, 2020), those most frequently encountered in the general population of patients, and supporting the initial medical decisions for hospitalisation and antibiotic treatment (Leber *et al.*, 2016; Soucek *et al.*, 2019; Tansarli and Chapin, 2020). Such a POC diagnosis, which is routinely made using nested or semi-nested PCRs, is prone to cross-contamination resulting in false-positive diagnoses (Boudet *et al.*, 2019; Vincent *et al.*, 2020). Current POC diagnosis also relies on the detection of a limited number of pathogens, and may thus miss microorganisms acting as life-threatening pathogens in selected populations, such as *Cryptococcus* in HIV-infected patients, in whom *Cryptococcus* most frequently causes fungal meningitis, with 223,000 new cases each year and 81% mortality (Ahn *et al.*, 2017; Xing *et al.*, 2019) and amoebas, such as *Acanthamoeba* (Behera *et al.*, 2016; Greninger *et al.*, 2015). The precise characterisation of such detected pathogens, including genotyping and anti-infectious susceptibility profiling, are pieces of medical information which are relevant for the immediate medical management of patients and contacts, and source tracing.

Metagenomic next generation sequencing (mNGS) directly applied to cerebrospinal fluid (CSF) emerged less than ten years ago, in the specific context of healthcare-associated meningitis (Naccache *et al.*, 2015), as an alternative to the laboratory diagnosis of CAM, potentially surpassing above-mentioned limitations of the current multiplex PCR approach, as discussed below (**Table 1**). However, no

review papers have been published including recommendations and the routine diagnostic implications of this approach. To update existing knowledge about the direct diagnosis of CAM by metagenomics, we conducted a literature search for studies applying mNGS directly to CSF for the diagnosis of CAM.

METHODS

Literature search. This study was conducted in accordance with the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) guidelines (Moher *et al.*, 2015). A systematic bibliography search was conducted on PubMed, Google Scholar, Web of Science, Microsoft Academic, Crossref and Semantic Scholar databases for studies published in English between 1 January 2015 and 31 December 2021 and related to the diagnosis of CAM by metagenomic NGS. Duplicate studies were removed in a first screening and the remaining papers were further screened based on title and abstract, according to the eligibility criteria. After full-text screening, only studies that met the inclusion criteria were included in this review, using the keywords: "metagenomic", “diagnosis”, "meningitis", “encephalitis", “next generation sequencing” and “cerebrospinal fluid”. These keywords were used in combination to perform an exhaustive search, as presented in **Figure 1**.

Eligibility and data. Studies with the following criteria were included in this review: Studies using mNGS-CSF for: 1) case reports; 2) prospective series; 3) retrospective series for non-routinely detected pathogens” (**Table 1, Supplementary data**). Review articles, studies performed on animals, non-clinical studies, and benchmarking studies were not included. Data extracted from selected studies included: first author name and year of publication, authors’ country, nucleic acid

extraction method including commercially available kits, mNGS platform and sequencer instrument, pipeline data analysis and software, and reference database used for pathogen genome detection of identified microorganisms. Included were the total number and discordance/agreement of mNGS data with data yielded by routinely used methods, including molecular tests and culture. These data were reviewed and extracted after validation of the inclusion criteria by the authors.

RESULTS

Included studies. Bibliographic searches identified 3,341 articles and, following the Prisma diagram (**Figure 1**), 2,080 articles were removed after deduplication and 1,199 additional articles were removed by title and abstract screening. From the 62 remaining articles, 11 articles were removed after full-text reading, including four review articles and seven non-relevant articles, resulting in 51 articles analysed in this review.

Study characteristics.

Participants. In 2015, the first reported application of mNGS directly on CSF (and brain biopsy) enabled the post-mortem diagnosis of Astrovirus-related encephalitis in an immunocompromised patient (Naccache *et al.*, 2015). A seven-year review of publications indicated only nine studies published between 2015 and 2018, ten in 2019 and 42/51 (82%) publications between 2019 and 2021, including 22 (43%) publications in 2021 (**Figure 2**). As for the geographic origin of published studies, 28 (56%) were published in Asia, including 25 (49%) from Chinese laboratories (**Table 1**), one each from Saudi Arabia (Guan *et al.*, 2021), Bangladesh (Saha *et al.*, 2019)

and Japan (Kawada *et al.*, 2016) (6.1%); nine in the United States (**Table 1**); one in Mexico (Joanna María *et al.*, 2016); five in France (10%) (**Tabel 1**); two in the Netherlands (Edridge *et al.*, 2019; Carbo *et al.*, 2020); and one each in Switzerland, the United Kingdom, and Spain (Tschumi *et al.*, 2019; Manso *et al.*, 2020, Leon *et al.*, 2020). One study was also published in Guinea and another in Australia (Eibach *et al.*, 2019; Li *et al.*, 2021) (**Figure 2**).

Workflow.

Nucleic acid procedures. Only CSF investigations (apart from one post-mortem brain biopsy in the founding study (Neccache *et al.*, 2015)) were reviewed in this study. Depending on the prospective or retrospective nature of the investigations, fresh CSF was used in 63% of studies and frozen CFS in 37% of studies. DNA and RNA were manually extracted in 44% of studies, automatic extraction was performed in 20%, and 36% of studies did not report on the nucleic acid extraction method. The sample pre-treatment reported in 18/51 (35%) studies included centrifugation (~15,000 g), filtration and vortexing in 12 studies, DNase treatment in four studies (Edridge *et al.*, 2019; Manso *et al.*, 2020; Morsli *et al.*, 2021d; Piantadosi *et al.*, 2018)n and proteinase K treatment in five studies (**Table 1**) (Guan *et al.*, 2015; Miller *et al.*, 2019; Carbo *et al.*, 2020; Manso *et al.*, 2020; Morsli *et al.*, 2021a, 2021c; Piantadosi *et al.*, 2021), while no pre-treatment was performed in 15 studies and no such information was provided in 16 studies (**Table 1**). Extracted nucleic acids (retro-transcribed RNA and DNA) were always quantified using either a Qubit® fluorometer and a Qubit DNA and RNA High Sensitivity Assay Kit (Life Technology, USA) (**Table 1**) (Guan *et al.*, 2015; Miller *et al.*, 2019; Carbo *et al.*, 2020; Manso *et al.*, 2020; Morsli *et al.*, 2021a, 2021c; Piantadosi *et al.*, 2021), or NanoDrop spectrophotometer

(Thermo Fisher Scientific, USA) (Li *et al.*, 2021). Optimisation in the human-to-microbial ratio of DNA was achieved by intermediate microbial genome enrichment or human genome depletion (**Table 1**). DNase treatment was used to reduce human DNA in extracted RNA for mNGS investigations of RNA-viruses (**Table 1**) (Guan *et al.*, 2015; Miller *et al.*, 2019; Carbo *et al.*, 2020; Manso *et al.*, 2020; Morsli *et al.*, 2021a, 2021c; Piantadosi *et al.*, 2021), and bead-based capture kits were also used to remove human DNA (Miller *et al.*, 2019; Gao *et al.*, 2021; Piantadosi *et al.*, 2021, Leon *et al.*, 2020). Further, microbial genomes could be enriched by specific primer amplification for whole genome investigations (**Table 1**). cDNA and double-stranded synthesis were needed prior to library preparation when RNA was investigated (**Table 1)**, and in the case of RNA genome enrichment, an RT-one-step protocol was followed by reverse-transcription and pathogen genome amplification (Morsli *et al.*, 2021c). In two studies, DNA was mechanically broken prior to library construction (Hu *et al.*, 2018; Zhang *et al.*, 2019).

NGS library preparation and sequencing. Three main protocols were used for mNGS library preparation (**Table 1, Figure 3**). Illumina pair-end protocols and reagents were used for mNGS library preparation in 19/51 (37%) studies, including Nextera XT DNA Library Prep Kit in 16/51 (31%) and one each with the TruSeq Universal kit (Erdem *et al.*, 2021) and VAHTS Universal DNA Library Prep Kit (Chen *et al.*, 2020). Standard BGISEQ pair-end protocols were used in 6/51 studies. Two studies used the PACEseq mNGS test (Hugobiotech, Beijing, China) (Mao *et al.*, 2021; Zeng *et al.*, 2021), two studies used VIDISCA and Ion Torrent end-repair library (Edridge *et al.*, 2019; Gao *et al.*, 2021), and one study each used the NEBNext Ultra II Directional RNA Library prep kit (New England Biolabs, Ipswich, MA, USA) (Carbo *et al.*, 2020, Leon *et al.*, 2020), the KAPA Hyper Prep Kit (Kapa

Biosystem, Potters Bar, UK) (Yin *et al.*, 2021), the NuGEN Ovation Ultralow Library System V2 kit (NuGEN Technologies, USA) (Guan *et al.*, 2015) the Trio RNA-Seq kit (NuGEN Technologies) (Li *et al.*, 2021), and the Roche 454 GS FLX single-end library (Roche Diagnostics, Branford, CT) (Joanna María *et al.*, 2016). In three studies, the mNGS library was constructed following the single-end Oxford Nanopore library preparation protocol (Morsli *et al.*, 2022; Morsli *et al.*,2021a; Morsli *et al.*, 2021b). Finally, the library preparation protocol and kits were not reported in 14 studies (**Table 1, Figure 3**). mNGS libraries were sequenced using Illumina sequencers in 30/51 (59%) studies, MiSeq platform in 11/30, HiSeq platform in 8/30, Nextseq platform in 6/30, iSeq 100 platform in 4/30, and 3/30 studies used the NovaSeq platform. BGISEQ platforms were used in 12/51 (24%) of studies (**Table 1**), including BGISEQ-50, 100 and 500 platforms, while three studies used the Roche 454 GS FLX Titanium system platform, Ion PGM™ System platforms and Ion Torrent Proton Sequencer (Life Technologies, USA), respectively (Joanna María *et al.*, 2016; Edridge *et al.*, 2019; Gao *et al.*, 2021). Real-time sequencing with the Oxford Nanopore MinION sequencer was used in three studies (Morsli *et al.*, 2022; Morsli *et al.*, 2021a; Morsli *et al.*, 2021b) and no information about the sequencer was reported in five studies (**Table 1**). The use of Nanopore technologies, i.e. single-long-read sequencing platforms combined with direct read blasting against the NCBI GenBank database using EPI2ME online software (https://epi2me.nanoporetech.com/) or against an internal database using in-house pipelines, allowed for pathogen identification within the first minutes of sequencing (Morsli *et al.*, 2021a). Furthermore, all additional pieces of information such as bacteria profiling and genotyping, was obtained in less than six hours (Morsli *et al.*, 2022; Morsli *et al.*, 2021a; Morsli *et al.*, 2021b).

Sequence data analysis. NGS data quality was controlled using FastQC (Erdem *et al.*, 2021; Yin *et al.*, 2021), and the human genome could be removed by mNGS reads mapping to the *Homo sapiens* reference genome (*hg*19) using Burrows-Wheeler Alignment (BWA) in 40% of studies. Non-mapped reads could be assembled using Spades and De Novo assembly or directly against the reference genome using the BWA and CLC Genomic workbench (**Table 1**). For an exhaustive pathogen genome investigation, mNGS data were blasted (BLAST: Basic Local Alignment Search Tool (nih.gov)) against the NCBI GenBank database or against an in-house microbial genome database, regardless of the sequencing technology, yet with different timing as for Illumina and Nanopore technologies, as mentioned above (**Table 1**). The sequence-based ultra-rapid pathogen identification computational pipeline (SURPI) was a powerful method for pathogen identification, combining blast and mapping of both pathogen genome and protein encoding sequences directly from mNGS outputs (Greninger *et al.*, 2015; Miller *et al.*, 2019). When only one specific aetiology was investigated, a reference database constructed with such specific pathogen genomes and proteins could be used for its accurate identification (Piantadosi *et al.*, 2017; Manso *et al.*, 2020). Depending on the previous pathogen identification, a specific pathogen genome was extracted directly as consensus sequences in FASTA files, after mapping to the reference genome directly from CLC and BWA software for further analysis (**Table 1, Figure 3**). Virus genotyping based on hit-blast identified strains could be confirmed by phylogenetic analysis based on sequence similarity level, while bacteria genotyping could be predicted online by Multi-Locus Sequence Typing online platform (**Figure 3**). Also, the in silico antibiotic resistance pattern could be predicted by aligning bacterial genome sequences against online databases of antibiotic-resistance encoding genes (**Table 1**) (Guan *et*

al., 2015; Miller *et al.*, 2019; Carbo *et al.*, 2020; Manso *et al.*, 2020; Morsli *et al.*, 2021a, 2021c; Piantadosi *et al.*, 2021), mostly represented by ResFinder on the Centre for Genomic Epidemiology server (Bortolaia *et al.*, 2020; Morsli *et al.*, 2022; Morsli *et al.*, 2021a; Morsli *et al.*, 2021b).

Pathogen Detection and Characterisation. Of the 51 studies analysed, a total of 1,248 CSF samples collected from CAM patients were investigated in parallel by mNGS reference and routine molecular diagnosis and culture. Routine methods and mNGS yielded concordant results in 1,031 (82.6%) CSF samples, including no pathogen documentation in 566 CSF samples and concordant identification in 465 CSF samples. Discordant results were observed in 217 (17.4%) CSF samples, including 87 CSF samples documented by routine methods only, 103 CSF samples documented by mNGS only and 27 CSF samples in which documented microorganisms differed from routine methods and mNGS. Altogether, the 116 detected microorganisms included 63 bacteria, 38 viruses, 10 fungi and five parasites. mNGS detected 106/116 (91.4%) different microorganisms, including 50 (43.1%) detected by mNGS only, whereas 56 microorganisms were detected by both routine methods and mNGS. In addition, five microorganisms were detected by routine molecular diagnosis only, four microorganisms by culture only and one *Enterococcus gallinarum* strain was diagnosed by routine molecular diagnosis and culture (**Figure 4**). Further, there was concordant documentation of 56 different pathogens, including 30 bacterial pathogens, 19 viral pathogens, six fungi and one *Balamuthia mandrillaris* (*B. mandrillaris*) as the only amoeba detected in a CSF sample (**Table 1, Figure 4**). Of the 217 CSF samples in which mNGS investigation yielded results discordant with the routine method, mNGS conclusively found a pathogen in 38/103 CSF samples in which routine investigations found no pathogen,

as reported in 25/51 studies: mNGS over-detected RNA viruses, including *Human rhinovirus* and *Human coronavirus* (Li *et al.*, 2021), *Coxsackievirus* 9 and Mumps virus (Kawada *et al.*, 2016), *Saint-Louis Encephalitis virus* (Chiu *et al.*, 2017), *Powassan Virus* (Piantadosi *et al.*, 2018), *Toscana virus* (Tschumi et al., 2019), *Jamestown Canyon virus* (Solomon *et al.*, 2021), Enterovirus A71 (Leon *et al.*, 2020), and *Hepatitis E virus* (Carbo *et al.*, 2020), while only HSV-1, HHV-6 and EBV were identified as DNA viruses (Zhang *et al.*, 2019b; Carbo *et al.*, 2020). Bacterial pathogens documented in 11% of these cases included *Listeria monocytogenes* in four cases (Lan *et al.*, 2020; Yaoa *et al.*, 2016), *Ureaplasma parvum* in three cases (Wang *et al.*, 2020; Xing *et al.*, 2021; Zhan *et al.*, 2021) and one case each was detected of *Klebsiella pneumoniae* (Zeng *et al.*, 2021), *Pasteurella multocida* (Morsli *et al.*, 2022), *Enterococcus faecalis* (Zhang *et al.*, 2021a), *Nocardia farcinica* (Zhang *et al.*, 2021b), *Streptococcus suis* (Zhang *et al.*, 2021) and *Psychrobacter* sp. (Joanna María *et al.*, 2016). *Toxoplasma gondii, B. mandrillaris* and *Naegleria fowleri* parasite species were identified in four patients (*N. fowleri* in two patients) in whom routine diagnostic methods completely missed *N. fowleri* (Guan *et al.*, 2021; Hu *et al.*, 2018; Huang *et al.*, 2021; Wu *et al.*, 2020), while *Coccidioides posadasii* was the single fungal infection exclusively detected by mNGS in one patient with no previously documented infectious meningitis (Mao *et al.*, 2021) (**Supplementary data, Figure 4**).

Limitations. A review of published data indicated that in 4/217 cases, mNGS failed to detect a pathogen when the pathogen had been detected by RT-PCR with a Ct >37, mainly due to a low pathogen inoculum (Miller *et al.*, 2019), as well as for a pathogen detected by immunodetection assay (Xing *et al.*, 2021). These limitations could be due to:

1) Storage conditions: storage of the CSF at temperature above -80ºC may cause nucleic acid molecule damage. Moreover, lack of proper storage in the presence of RNase inhibitors, will likely result in RNA viruses undergoing some degradation.

2) Limited CSF volume affecting the DNA quantity used for NGS library preparation (Miller *et al.*, 2019), especially for the Oxford Nanopore libraries, which require 1 µg of DNA (Morsli *et al.*, 2021a; Morsli *et al.*, 2021b);

3) A CSF leukocyte count (> 10^3/mL) increasing the ratio of human genome, which required microbial genome enrichment (Guan *et al.*, 2015; Miller *et al.*, 2019; Carbo *et al.*, 2020; Manso *et al.*, 2020; Morsli *et al.*, 2021a; Morsli *et al.*, 2021c; Piantadosi *et al.*, 2021), and human genome depletion (**Table 1**). DNase treatment usually used to reduce human DNA in the extracted CSF can, therefore, generate false negative results, due to the degradation of the DNA pathogens (Edridge *et al.*, 2019). Alternatively, one limitation is related to the elimination of human DNA prior tosequencing, such as CSF pathogens whose genome is partially or totally integrated into the human genome, such as the case of HHV-6 (Edridge *et al.*, 2019). Library preparation and cross-contamination removal during sample preparation is essential to avoid any confusion of detection and interpretation, especially when working on several samples in the same run (**Table 1**) (Guan *et al.*, 2015; Miller *et al.*, 2019; Carbo *et al.*, 2020; Manso *et al.*, 2020; Morsli *et al.*, 2021a, 2021c; Piantadosi *et al.*, 2021). Depth, coverage, and sequence quality may be influenced by the sequencing platforms, as well as the kit used for library preparation, which is relative to the initial pathogen load in the CSF (**Table 1**) (Guan *et al.*, 2015; Miller *et al.*, 2019; Carbo *et al.*, 2020; Manso *et al.*, 2020; Morsli *et al.*, 2021a, 2021c; Piantadosi *et al.*, 2021), depending on the number of sequencing cycles for paired-end sequencing and the

read size for single-end long read sequencing. Assembly and data analysis mainly depend on the quantity and quality of reads generated by sequencers (Morsli *et al.*, 2021a; Morsli et al 2021c; Morsli *et al.*, 2021d). Pathogen identification is mainly based on in-house databases constructed with a limited number of pathogen genomes and species downloaded from the NCBI GenBank (**Table 1**) used for blast and mapping, which needs regular updating for the exhaustive identification of all possible microbial genomes detected in the CSF sample (**Table 1**). Performance analysis requires powerful software and pipelines to blast all mNGS data against the GenBank database, which is limited by cost and accessibility, such as the EPI2ME software used for real-time analysis of sequencing data generated by Nanopore Platforms (Morsli *et al.*, 2022; Morsli *et al.*, 2021a; Morsli *et al.*, 2021b).

DISCUSSION

Direct mNGS appears to be a relevant alternative diagnostic approach to simplex and multiplex RT-PCRs for the POC diagnosis of CAM. mNGS adds one-shot pieces of medically relevant information, including genotyping and antimicrobial susceptibility profile, in comparison to current PCR-based methods (Morsli *et al.*, 2021a; Morsli *et al.*, 2021b; Zhou *et al.*, 2021). The suitability of this new approach should be evaluated in all laboratories already providing molecular detection of pathogen-genome sequences in the CSF of patients with meningitis, and as a new technique in new POC laboratories bypassing previous multiplex RT-PCR (Boudet *et al.*, 2019; Morsli *et al.*, 2022; Vincent *et al.*, 2020). The basis for such an evaluation includes the flow of samples and cost of mNGS relative to multiplex PCR. Indeed, mNGS also provides an antimicrobial susceptibility profile to guide medical

management of the patient (Morsli *et al.*, 2022; Morsli *et al.*, 2021a; Morsli *et al.*, 2021b) and genotyping to detect outbreaks and to guide source tracing compared to multiplex PCRs (Broberg *et al.*, 2018). Such genotyping information may be medically relevant and associated with a particular prognosis of CAM (case of a few Enteroviruses) and certainly relevant to source tracing in the context of clustered cases and outbreaks. Compared to the limited number of the pathogens investigated by routine multiplex RT-PCR, mNGS identified 106 different microorganisms in CSFs from patients with meningitis, i.e. covering 91.4% of total microorganisms identified here. This allowed the detection of pathogen genomes in 38 non-routinely documented CSFs. In addition, mNGS can operate within infinitely greater open databases than those currently supporting available multiplex RT-PCR assays, which rely on fewer than 30 such entries, whereas genome sequence databases such as NCBI consist of more than 83,124 complete microbial genomes (including bacteria, viruses, fungi, and parasites), offering opportunities for the diagnosis of rare pathogens, as reviewed in this study.

Finally, a few current limitations of the mNGS approach are opportunities for improvements from the perspective of routine use. Most enzymes used in the above-described sequencing protocols were issued from cloning in competent bacteria such as *Escherichia coli* (*E. coli*) (Morsli *et al.*, 2021a), meaning potential contamination by *E. coli* DNA and a risk of a false-positive diagnosis of *E. coli* meningitis. As an illustration, Nanopore sequencing results regularly consisted of *E. coli* DNA resulting from the recommended internal library control, and *Shigella* and T4 phage reads issued from repair and ligation enzymes (Morsli *et al.*, 2022; Morsli *et al.*, 2021a; Morsli *et al.*, 2021b). This limit could be overcome by replacing the *E. coli* control and using the human genome as an internal control, in line with our own practice. The

quantity of data generated by sequencing was sometimes insufficient to support an accurate interpretation, requiring additional enrichment and human genome depletion steps to improve the sensitivity and specificity of detection of CAM causative agents (Carbo *et al.*, 2020; Gao *et al.*, 2021; Miller *et al.*, 2019; Morsli *et al.*, 2021c; Piantadosi *et al.*, 2021). Moreover, sequence variation linked to high mutational levels, as observed in RNA viruses including Enterovirus (Piantadosi *et al.*, 2017; Morsli *et al.*, 2021d; Morsli *et al.*, 2021c), with more than 300 genotypes (Isaacs *et al.*, 2018), and obscuring the identification capability of the mNGS approach, further indicates the necessity for pathogen genome enrichment to achieve appropriate sequencing depth. This is usually limited by the CSF volume which makes it not possible to extract the DNA and RNA separately (Li *et al.*, 2021). The sensitivity of mNGS depended on the nature of the causative pathogen, being lower than routine techniques for bacterial and fungal meningitis, at 73.3% and < 85% respectively (Xing *et al.*, 2020; Xing *et al.*, 2021b). In contrast, mNGS appeared to be highly sensitive (>90%) in cases of mycobacterial and viral meningitis (Kawada *et al.*, 2016; Miller *et al.*, 2019; Xing *et al.*, 2020; Zhang *et al.*, 2021b). However, human genome depletion by DNase treatment can produce false-negative results in the case of RNA investigation, by degrading the pathogen DNA (Edridge *et al.*, 2019; Wilson *et al.*, 2019).

The mNGS approach is not currently standardised and remains in its infancy. Microbiologists may, therefore, wish to develop different strategies to investigate RNA and/or DNA pathogens, relying on available mNGS materials and reagents as well as mNGS platform, and in-house pipelines using local epidemiology-driven, specific pathogen genome databases (**Figure 3**). Finally, because this identification is database-dependent, attention should be given to curating and updating local

databases (Carbo *et al.*, 2021; Ji *et al.*, 2020; Xing *et al.*, 2021). In contrast to routine tests based on nested and semi-nested PCRs generating up to 25% false-positive results (Boudet *et al.* 2019), the mNGS approach does not involve any prior amplification and no specific pathogen target, thus limiting the opportunity for such false-positives. Additional information about genotyping and in silico antibiotic susceptibility testing was added, even in cases of uncultured bacteria.

As a time and cost-effective approach, real-time mNGS could be implemented in POC laboratories for the diagnosis of CAM, especially with regards to undocumented meningeal disease (Morsli *et al.*, 2022).

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Conflicts of Interest

The authors have no conflicts of interest to declare, and the authors received no fees or benefits from any of the suppliers referenced in this review.

Ethical approval.

Not applicable.

Authors' contributions.

MM: Literature search, data collection, data cleaning, data interpreting, validation and writing of the manuscript. LJP and DM, design of the study, data interpreting, validation, funding, critically reviewing the manuscript, coordination, and direction of the work. The data presented here were extracted by MM and validated by JPL and MD after review.

All authors declare that they have read and approved the manuscript.

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Figures

Figure 1: Prisma diagram of eligible articles recorded after removing duplications. Six bibliographic databases were reviewed using the following key words: "metagenomic", “diagnosis”, "meningitis", “encephalitis", “next generation sequencing” and “cerebrospinal fluid”, used alone and/or in combination. Only 51 articles were eligible for this review, after screening according to the inclusion criteria described above.

Figure 2: 1) Number and geographical distribution of publication records after duplication removed**. 2)** Publication chronology according to the used sequencing technologies.

Figure 3: Metagenomic Next-Generation Sequencing workflow. Upon reception of the CSF, mechanical and enzymatic sample pre-treatment was performed before manual or automatic DNA/RNA extraction. Before mNGS library preparation, the human genome was removed with or without microbial genome enrichment, then mNGS libraries were prepared following pair-end or single-end long read protocol, depending on the sequencing platform. Pathogen genome identified directly by blast against NCBI GenBank and/or local microbial genome database downloaded from GenBank, using in-house pipelines. In-silico antibiogram and genotyping were performed depending on the nature of the identified pathogenic agent.

Figure 4: SAM pathogens identified by routine molecular diagnosis, culture, and mNGS. A total of 116 different microorganisms were identified, including 50 (43.1%) only by mNGS, 43 by both routine molecular diagnosis and mNGS, 12 by culture, mNGS and routine molecular diagnosis, 10 by culture and mNGS, five only by

routine molecular diagnosis, four only by culture, and one only by culture and routine molecular diagnosis

643 **Table 1**: metagenomics pipelines.

Reference	Country	Category	Sample type	Total patient	Sample preparation	DNA/RNA extraction	Microbial genome enrichment	Library preparation	Instrument	Software	Database
(Greninger et al., 2015)	California, USA	Case report	Fresh CSF	1	No	EZ1 Viral kit (Qiagen)	Turbo DNase (Ambion)	Nextera XT protocol (Illumina)	Illumina MiSeq	SURPI pipeline	NCBI GeneBank database
(Guan et al., 2015)	Tianjin, China	Prospective study	Frozen CSF	4	No	TIANamp Micro DNA Kit (TIANGEN BIOTECH)	Sigma-Aldrich WGA4 Kit (WGA)	Not available	BGISEQ-100 platform	Burrows-Wheeler Alignment, SoapCoverage software	Microbial Genome Databases
(Ming Yaoa* et al., 2016)	Beijing, China	retrospective study	Frozen CSF	3	No	TIANamp Micro DNA Kit (Tiangen Biotech)	No	Not available	BGISEQ-100	Burrows-Wheeler Alignment	Microbial Genome Database
(Joanna María et al., 2016)	Mexico	Case report	Frozen CSF	1	No	MagNA Pure LC 2.0 instrument, Total Nucleic Acid isolation kit (Roche)	TransPlex Whole Transcriptome Amplification kit WTA, (Sigma-Aldrich) and GenomiPhi V2 (Healthcare Life Sciences)	Roche 454 GS FLX single-end library	454 GS FLX Titanium system (Roche)	GS de novo Assembler version 2.6,	NCBI GenBank
(Kawada et al., 2016)	Nagoya, Japan	Prospective study	Fresh CSF	18	0.45-μ m filter (Merck-Millipore, Temecula, CA)	QIAamp Viral RNA Mini kit (Qiagen)	Turbo DNase (Ambion, Darmstadt, Germany)	Nextera XT DNA Sample preparation kit and ScriptSeq v2 (Illumina)	Illumina MiSeq, Illumina HiSeq 2500	MEGAN5, CLC workbench	MEGABLAST
(Chiu et al., 2017)	California, USA	Case report	Fresh CSF	1	Not available	Not available	Not available	Not available	Illumina Miseq	SURPI+ computational pipeline	Microbial genome database

(Piantadosi et al., 2017)	Boston, USA	Case report	Fresh CSF	1	centrifugation 90 minutes/ 18,000 xg/4°C	QIAamp Viral Mini Kit (Qiagen)	TURBO DNase (Thermo Fisher)	Nextera XT	Illumina MiSeq	Kraken, De novo assembly	published computational pipeline viral-NGS
(Hu et al., 2018)	Nanjing, China	Case report	Fresh CSF	1	No	TIANamp Micro DNA Kit (TIANGEN BIO- TECH)	Sonication (Bioruptor Pico protocols)	end-repaired adaptation and PCR amplification (BGI, Tianjin, China)	BGISEQ-50 platform	Burrows-Wheeler Alignment	Microbial genome database
(Piantadosi et al., 2018)	Boston, USA	Case report	Fresh CSF	1	TURBO DNase (Thermo Fisher)	QIAamp Viral Mini Kit (Qiagen)	TURBO DNase (Thermo Fisher)	Nextera XT (Illumina)	Illumina MiSeq, HiSeq 2500	Kraken, Geneious version 8.1.7	NCBI GenBank Database
(Xing et al., 2019)	Beijing, China	Prospective study	Frozen CSF	12	Not available	Not available	Not available	Not available	BGISEQ-500/50 platform	Burrows-Wheeler Alignment	Microbial genome Database
(Tschumi et al., 2019)	Zurich, Switzerland	Case report	Fresh CSF	1	centrifugation and filtration	Not available	DNase treatment	Nextera XT (Illumina)	MiSeq	VirMet pipeline	NCBI GenBank database
(Saha et al., 2019)	Bangladesh	Retrospective study	Frozen CSF	91	Not available	Not available	Not available	Nextera XT (Illumina)	Illumina NovaSeq	IDseq bioinformatics pipeline	NCBI GenBank database
(S. Wang et al., 2019)	Beijing, China	Retrospective study	Frozen CSF	23	glass beads, vortexed 20 min, centrifuged at 8000 rcf	TIANamp Micro DNA Kit (TIANGEN BIOTECH)	No	end-repaired adaptation and PCR amplification (BGI, Tianjin, China)	BGISEQ-100	Mapping with WBA, BLAST	Microbial genome database
(Wilson et al., 2019)	California, USA	Retrospective study	Fresh CSF	204	Not available	Not available	Not available	Nextera XT (Illumina)	Illumina HiSeq	SURPI+ pipeline	NCBI GenBank database

(Zhang et al., 2019b)	Hebei, China	Case report	Fresh CSF	1	No	TIANamp Micro DNA Kit (Tiangen Biotech)	No	end-repaired adaptation and PCR amplification (BGI, Tianjin, China)	BGISEQ-100	Burrows-Wheeler Alignment	Microbial genome database
(Edridge et al., 2019)	Amsterdam, Netherlands	Retrospective study	Frozen CSF	45	Centrifugation TURBO™ DNase (Thermo Fisher)	Manually extracted Boom method	MseI (T^TAA; New England Biolabs)	VIDISCA library preparation	Ion PGM™ System	Taxonomer, CodonCode Aligner (version 6.0.2)	UBLAST
(Zhang et al., 2019a)	Beijing, China	Retrospective study	Fresh CSF	135	No	TIANamp Micro DNA Kit (DP316, Tiangen Biotech, Beijing, China).	No	BGISEQ-500 standard protocol	BGISEQ-500 sequencing	Burrows-Wheeler Alignment	Microbial genome database
(Miller et al., 2019)	California, USA	Retrospective study	Fresh CSF	95	FastPrep-24 bead beater (MP Biomedicals)	EZ1 Virus Mini Kit v2.0 (Qiagen)	NEB Microbiome Enrichment Kit (New England Biolabs) Turbo DNAse (Thermo-fisher)	Nextera XT DNA Library Prep Kit (Illumina	Illumina HiSeq	SURPI+ pipeline	GenBank reference database
(Eibach et al., 2019)	Agogo, Ghana	Retrospective study	Frozen CSF	70	No	MagMAX™ Viral RNA Isolation Kit (Life Technologies)	No	BGISEQ-500 standard protocol	Illumina MiSeq	CLC workbench, Trinity v2.6.6, Geneious v11, DIAMOND v0.9.6	NCBI GenBank database
(Chen et al., 2020)	Yangzhou, China	Case report	Fresh CSF	4	Centrifugation	TIANamp Micro DNA Kit (TianGen Biotech)	Ultrasonicator (Covaris)	VAHTS Universal DNA Library Prep Kit for Illumina V3 kit	Illumina NextSeq500	Burrows-Wheeler Alignment	Microbial genome database

(Lan et al., 2020)	Hunan, China	Case report	Fresh CSF	1	No	TIANGEN DNA Mini kit DP316 (Tiangen Biotech, Beijing, China)	No	Not available	Illumina NextSeq	Burrows-Wheeler Alignment	Microbial genome database
((Zhang et al., 2020)	Hunan, China	Case report	Fresh CSF	1	Not available	Not available	Not available	Not available	Not available	Not available	Not available
(Xing et al., 2020)	China	Prospective study	Frozen CSF	213	Glass beads, vortex	Not available	Not available	Not available	BGISEQ-500	Burrows-Wheeler Alignment	Microbial genome database
(Yan et al., 2020)	Shanghai, China	Prospective study	Frozen CSF	51	Glass beads, vortex 2800-3200RPM for 30 min	TIANamp Micro DNA Kit (TIANGEN BIOTECH)	No	end-repaired adaptation and PCR amplification (BGI)	BGISEQ-50 platform	Burrows–Wheeler Alignmen	Microbial Genome Databases
(Wang et al., 2020)	Wenzhou, China	Case report	Fresh CSF	1	Not available	Not available	Not available	Not available	BGISEQ platform	Burrows-Wheeler Alignment	Microbial Genome Databases
(Wu et al., 2020)	Shanghai, China	Case report	Fresh CSF	1	Not available	Not available	Not available	Not available	BGISEQ platform	Burrows-Wheeler Alignment	Microbial Genome Databases
(Manso et al., 2020)	London, UK	Prospective study	Fresh CSF	12	Filtration and Turbo DNAse treatment	PureLink Viral RNA/DNA Mini Kit (Invitrogen)	No	Nextera XT DNA library prep kit (Illumina)	Illumina MiSeq	Trimmomatic v0.39, PRINSEQ, mapping with PALADIN, BWA MEM	In-house database comprising the RefSeq viral protein sequences from NCBI
(Carbo et al., 2020)	Leiden, The Netherlands	Prospective study	Frozen CSF	41	No	MagNApure 96 DNA and Viral NA Small volume extraction kit (ROCHE)	SpeedVac vacuum con-centrator (Eppendorf), SeqCap EZ Hypercap probes (Roche)	NEBNext Ultra II Directional RNA Library prep kit (New England Biolabs)	Illumina NovaSeq6000	Illumina data analysis pipeline RTA3.4.4 and bcl2fastq v2.20, Genome Detective version 1.111	An index database constructed from NCBI

(Leon et al., 2020)	Barcelona, Spain	Retrospective study	Frozen CSF	20	No	Direct-zol RNA MicroPrep with TRI reagent (Zymo Research)	Depletion of Abundant Sequences by Hybridization (DASH)	NEBNext Ultra II Directional RNA Library prep kit (New England Biolabs)	Illumina HiSeq 4000 instrument	Geneious version 10.2.3, SPAdes version 3.10.0, MUSCLE, MAFFT.	Local database
(Solomon et al., 2021)	Boston, USA	Case report	Frozen CSF	1	Not available	Not available	Not available	Not available	Illumina HiSeq 2500	SURPI+ pipeline	NCBI GenBank database
(Li et al., 2021)	Sydney, Australia	Prospective study	Frozen CSF	18	No	RNeasy plus universal kit (QIAGEN)	No	Trio RNA-Seq kit (NuGEN Technologies, USA) was	Illumina NovaSeq platform	Blastn, Blastx, Diamond, Megahit	NCBI GenBank databases,
(Zhan et al., 2021)	Hangzhou, China	Case report	Fresh CSF	1	Not available	Not available	Not available	Not available	BGISEQ-500 sequencing	Not available	Not available
(Zhou et al., 2021)	Hunan, China	Case report	Fresh CSF	1	No	Not available	No	Nexter XT	NextSeq 550	Kraken	In-house database
(Mao et al., 2021)	Guangzhou, China	Case report	Frozen CSF	1	Not available	TIANamp Magnetic DNA Kit (Tiangen)	Not available	PACEseq mNGS (Hugobiotech)	NextSeq 550	Burrows-Wheeler Alignment	Microbial genome database
(Zhang et al., 2021a)	Hunan, China	Case report	Fresh CSF	1	Not available	Not available	Not available	Not available	Not available	Not available	Not available
(Huang et al., 2021)	Guangzhou, China	Case report	Fresh CSF	1	centrifugation 5 min at 15,000 rpm	magnetic beads [Sagene™, Guangzhou, CHINA]	No	Nexter XT	Illumina™ NextSeq 550 DX	Not available	Not available
(Xing et al., 2021a)	Hebei, China	Retrospective study	Fresh CSF	7	Not available	Not available	Not available	Not available	BGISEQ-500 sequencing	Burrows-Wheeler Alignment	Microbial genome database

(Erdem et al., 2021)	Ohio, USA	Prospective study	Frozen CSF	37	No	QIAamp Viral RNA Mini Kit (Qiagen)	No	TruSeq Universal kit (Illumina)	Illumina HiSeq4000	FastQC, Cutadpat and PRINSEQ tools, Bowtie2 mapper 2.0.6, CDHIT tool. de novo assembled using MIRA (v 4.0), MegaBLAST	NCBI GeneBank database
(Xing et al., 2021b)	China	Case report	Frozen CSF	1	Not available	Not available	Not available	BGISEQ-500 standard protocol	BGISEQ 50 MGI DNBSEQ	Burrows-Wheeler Alignment	Microbial genome database
(Yin et al., 2021)	Jiangsu, China	Retrospective study	Frozen CSF	1	No	TIANamp Magnetic DNA Kit (Tiangen)	No	KAPA Hyper Prep Kit (KAPA Biosystems) according	Illumina NextSeq 550Dx	Trimmomatic v.0.36 software, Bowtie2 software. Kraken 2 software	Microbial genome database
(Zeng et al., 2021)	Changsha, China	Case report	Fresh CSF	1	Not available	Not available	Not available	PACEseq mNGS test (Hugobiotech)	Illumina NextSeq 550	Not available	Not available
(Guan et al., 2021)	Riyadh, Saudi Arabia	Case report	Frozen CSF	1	No	DNeasy Blood & Tissue Kits (QIAGEN)	No	NuGEN Ovation Ultralow Library System V2 (NuGEN)	Illumina HiSeq 4000, Illumina iSeq 100	MEGAHIT assembler v1·1·4, BBmap with 0·98, Metabat v2·12·1, Spades	NCBI GenBank database
(Zhang et al., 2021b)	Zhejiang, China	Case report	Fresh CSF	1	Not available	Not available	Not available	Nexter XT	Illumina NextSeq	Kraken	Kraken microbial database
(Piantadosi et al., 2021)	Boston, USA	Prospective study	Fresh CSF	68	No	QIAamp viral RNA minikit (Qiagen)	NEBNext microbiome DNA enrichment kit (New England BioLabs)	Nextera XT DNA library prep kit (Illumina)	Illumina MiSeq	KrakenUniq, BLAST	NCBI GenBank database

(Morsli et al., 2021d)	Marseille, France	Case report	Fresh CSF	1	Proteinase K 20 minutes	QIAamp Viral RNA Mini Kit solutions (Qiagen)	Turbo DNase (Thermo Fisher) and	Nextera XT V2	Illumina iSeq 100	Spade, BLAST, CLC workbench	NCBI GenBank database
(Fan et al., 2021)	Guangzhou, China	Retrospective study	Frozen CSF	11	Not available	Not available	Not available	Not available	BGISEQ platform	Burrows-Wheeler Alignment	Microbial Genome Databases
(Morsli et al., 2021c)	Marseille, France	Case report	Fresh CSF	1	Proteinase K 20 minutes	Virus Mini Kit v2.0 (Qiagen	Spiked primer enrichment	Nextera XT V2	Illumina Miseq, iSeq 100	Spade, BLAST, CLC workbench	NCBI GenBank database
(Morsli et al., 2021b)	Marseille, France	Case report	Fresh CSF	1	Proteinase K 20 minutes	EZ1 DNA Kit (Qiagen)	No	Oxford Nanopore MinION library preparation	Oxford Nanopore MinION	EPI2ME, Kraken-2, Pavian	NCBI GenBank database
(Morsli et al., 2021a)	Marseille, France	Case report	Fresh CSF	1	Proteinase K 20 minutes	EZ1 DNA Kit (Qiagen)	No	Oxford Nanopore MinION library preparation	Oxford nanopore MinION, Illumina iSeq	EPI2M2, Spades, CLC genomic workbench	NCBI GenBank database
(Gao et al., 2021)	Beijing, China	Prospective study	Fresh CSF	38	centrifugation 10 min at 13,000 rpm	QIAamp DNA Microbiome Kit (Qiagen)	NEBNext microbiome DNA enrichment kit (New England BioLabs)	Nextera XT kit v2 Ion Torrent end-repair library	Illumina MiSeq, Ion Torrent Proton	Burrows-Wheeler alignment was	Microbial genome databases
(Morsli et al., 2022)	Marseille, France	Case report	Fresh CSF	1	Proteinase K 20 minutes	EZ1 DNA Kit (Qiagen)	No	Oxford Nanopore MinION library preparation	Oxford Nanopore MinION	EPI2ME and CLC Genomics Workbench, software	NCBI GenBank database

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Figure 1.TIF

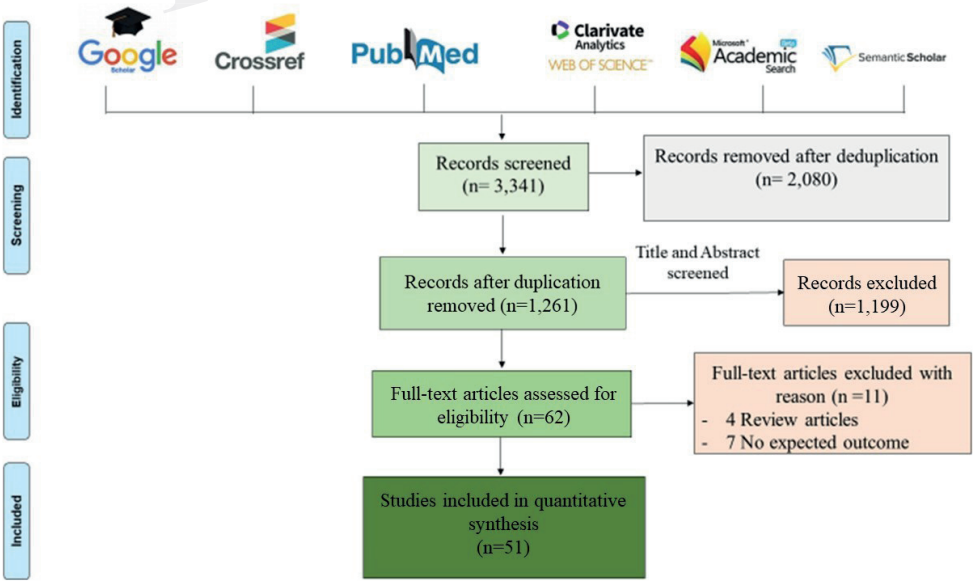
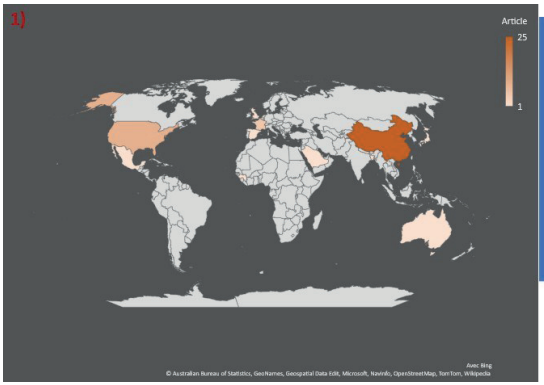


Figure 2.TIF



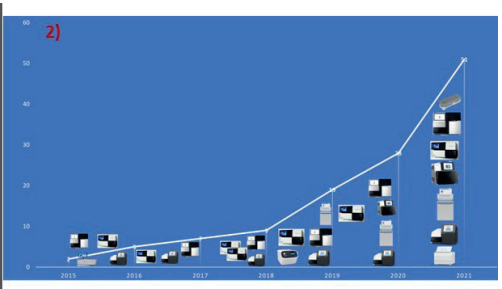


Figure 3.TIF

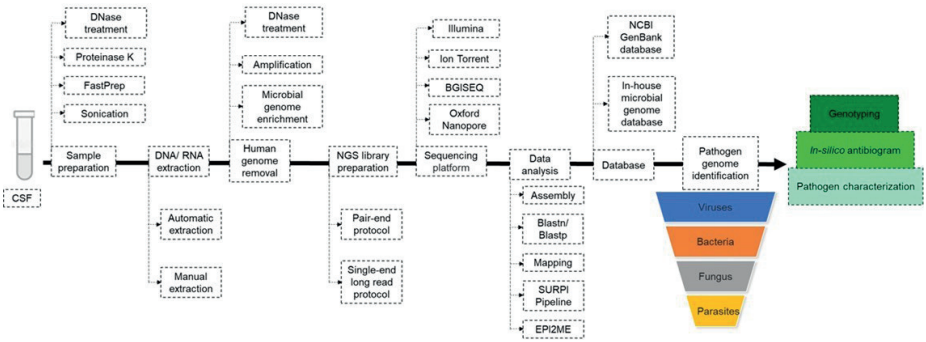
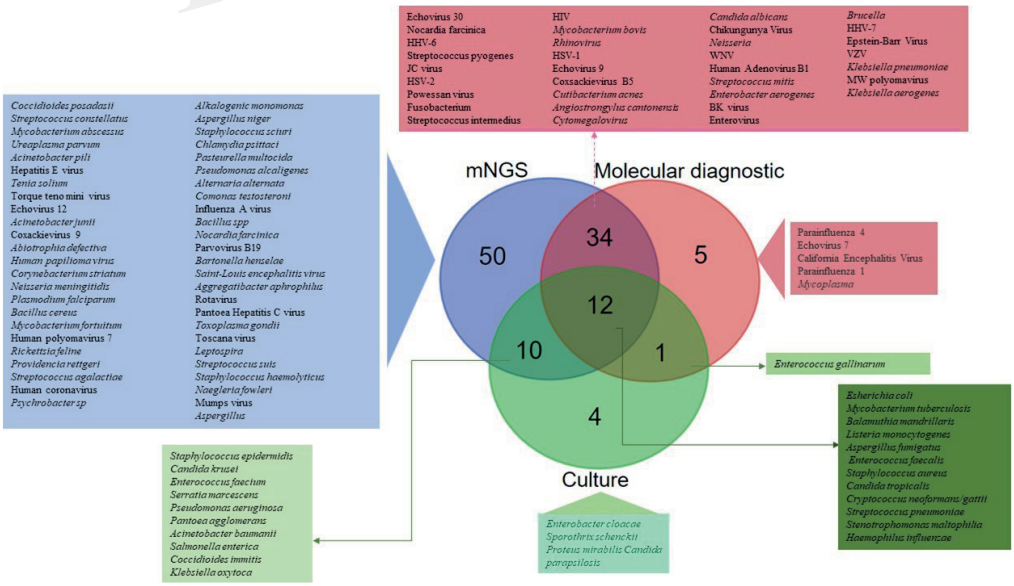


Figure 4.TIF



Chapitre II : Etude Rétrospective des méningites communautaires à Marseille et à Nîmes entre 2014-2019.

Préambule.

Les études épidémiologiques des méningites infectieuses publiées concernent toutes les cas documentés, et plus particulièrement l'épidémiologie des méningites communautaires dues à un pathogène donné dans un lieu donné durant une période donnée; permettant la comparaison de prévalence d'un pathogène par rapport à un autre selon la localisation géographique, la population et parfois la saison (14-17,19,48–51). En Europe par exemple, chaque année une épidémie d'Entérovirus est enregistrée en été, plus prévalente chez les enfants et les individus de bas âge (8,48,52). Ainsi, plus de 4.537 cas ont été rapportés dans 15 pays Européens entre Janvier et Septembre 2018, affectant préférentiellement les nouveau-nés et les personnes âgées de 26 à 45 ans (8). En Europe également, les infections bactériennes sont moins fréquentes que les méningites virales mais sont plus invasives et souvent associées à la saison froide et des populations âgées (53–57).

Après accord préalable du Comité d'Éthique de l'IHU Méditerranée Infection et de l'Interface Recherche Bioéthique Institutional Review Board du CHU Nîmes, nous avons conduit une étude rétrospective anonymisée des méningites communautaires diagnostiquées par les laboratoires de microbiologie clinique des centres hospitalo-universitaires de Nîmes et de Marseille, sur une période de 61 mois. Au total 20.779 échantillons de LCR collectés chez 20.779 patients ont été inclus dans cette étude après une sélection des cas strictement communautaires, dédoublonnés. Tous les cas associés aux soins incluant, neurochirurgie, dialyse, transplantation et hospitalisation de plus de 48 heures ont été exclus de cette étude. Après organisation des données patients anonymisées selon l'âge, le sexe, le centre et l'étiologie, ces données ont été analysées par des tests statistiques sur le logiciel R version (3.6.1) (58). Ensuite, nous avons synchronisé les données épidémiologiques des deux villes et les données climatiques par des séries temporelles afin de définir les épidémies et d'identifier une éventuelle corrélation entre les épidémies la saison et le climat.

Notre analyse portant sur un total de 20.779 patients entre décembre 2014 et décembre 2019, dont 15.246 patients à IHU Méditerranée Infection de Marseille et 5.533 patients au CHU Nîmes, a confirmé les données précédemment publiées, retrouvant uniquement 2.209 (10,63%) de cas étaient documentés pour un total de 62 microorganismes identifiés dans cette série. Mais l'originalité de notre travail réside dans l'analyse dynamique dans le temps (5 années) et dans l'espace (2 laboratoires situés à environ 120 kilomètres) des cas de syndrome méningé communautaire et de leurs étiologies infectieuses, montrant que 12.919/20.779 (62,2%) des cas étaient inclus dans 10 épisodes épidémiques, avec un taux de documentation de 11,3% au cours des

épidémies contre 10.1% hors épidémie; les épidémies de syndrome méningé communautaire se déplaçant toujours de Nîmes vers Marseille (c'est à dire d'Ouest vers l'Est), à une vitesse moyenne de 09 km/jour; montrant une corrélation significative avec le déplacement dans la même direction des pics de température.

Ce travail original nous a indiqué des épidémies de syndrome méningé communautaire non documentées, suggérant d'investiguer en deuxième ligne des explorations de première ligne, les prélèvements de LCR conservés en biobanque, pour tenter de démasquer le microorganisme ou les microorganismes responsables. L'ensemble de ces données sont rapportées dans l'article ci joint qui est soumis pour publication à Infection.

Article 2

Community-Acquired Meningitis Syndrome Outbreaks, Southern France.

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Medicine

Community-Acquired Meningitis Syndrome Outbreaks, Southern France.

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Abstract:	In southern France, community-acquired meningitis syndrome (CAM) cases are typically clustered as outbreaks whose determinants remain unknown. This 61-month retrospective investigation in Nîmes and Marseille university hospital laboratories, yielded 2,209/20,779 (10.6%) documented CAM cases caused by 62 different pathogens, represented by seasonal viral etiologies (78.8%), including enterovirus, Herpes Simplex Virus (HSV) and Varicella-Zoster Virus (VZV) (1,620/2,209=73.4%). Multi Correspondence Analysis revealed an association of infection with age and sex, with the risk of infection relatively higher in young men, as confirmed by Fisher's exact test ($p<10^{3}$). Bacterial meningitis accounted for 20% of cases, mostly caused by Streptococcus pneumoniae in 27.4% of cases, Neisseria meningitidis in 12.5% and Haemophilus influenzae in 9.5% with bacteria/virus coinfection in 0.9%, and only six cases of documented fungal meningitis. In total, 62.6% of cases of which 88.7% were undocumented, arose from 10 outbreaks; with 33.2% of undocumented cases were aged > 60 years vs. 19.2% of documented cases (P <0.001), and viral infection was more common in the summer (87.5% in summer vs. 72.3% in other seasons, P <0.001). Outbreaks most often started in Nîmes and moved eastward toward Marseille at a ~9 km/day speed, and these dynamics significantly correlated with the selected temperature, especially during summer outbreaks; particularly, incidence of enterovirus-driven outbreaks correlated with temperature with correlation coefficients of 0.64 in Nîmes and 0.72 in Marseille, and its occurrence in Marseille lagged that in Nîmes by 1-2 weeks. Tracing CAM outbreak dynamics during this retrospective investigation in southern France yielded a speed of displacement that correlated with the variation in temperature between both cities, and these results provide clues for the next occurrence of undocumented outbreaks.

Community-Acquired Meningitis Syndrome Outbreaks, Southern France.

Morsli Madjid [1, 2 †], Salipante Florian [3 †], Kerharo Quentin [4], Boudet Agathe [5], Stephan Robin [5], Dunyach-Remy Catherine [5], Zandotti Christine [1, 4], Lavigne Jean-Philippe [5], Drancourt Michel [1, 2, 4]

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Key words: Community-acquired meningitis (CAM), cerebrospinal fluid (CSF), etiology, outbreak, nondocumented meningitis, season, dynamics.

Abstract

Background In southern France, community-acquired meningitis syndrome (CAM) cases are typically clustered as outbreaks whose determinants remain unknown.

Methods A 5-year retrospective investigation of CAM cases routinely diagnosed in two university hospital laboratories located 120 km apart in Nîmes and Marseille, southern France, was conducted. Relationships between CAM diagnosis (negative or undocumented vs. positive or documented cases), patient age and sex, and the season, causative pathogens and period of outbreak were screened with multiple correspondence analysis (MCA). Relationships between variables incorporating outbreak location (Nîmes/Marseille) and season and patient sex and age were further analyzed with Pearson's chi-square test for qualitative variables and the Wilcoxon-Mann–Whitney test for quantitative variables.

Results Over 61 months, 2,209 of 20,779 (10.6%) documented CAM cases were caused by 62 different pathogens, including the most prevalent seasonal viral etiologies (78.8%), which are mostly represented by enterovirus, *herpes simplex virus* (HSV) and *varicella-zoster virus* (VZV) (1,620/2,209=73.4%); the results of MCA revealed an association of infection with age and sex, with the risk of infection relatively higher in young men than in other groups, as confirmed by Fisher's exact test ($p<10^3$). Bacterial meningitis accounted for 20% of cases and was caused by *Streptococcus pneumoniae* in 27.4% of cases, *Neisseria meningitidis* in 12.5% and *Haemophilus influenzae* in 9.5%. Bacteria/virus coinfection occurred in 0.9% of cases, and only six cases of fungal meningitis were documented. In total, 62.6% of cases arose from 10 outbreaks, of which 88.7% were undocumented; patients were aged > 60 years in 33.2% of undocumented cases vs. 19.2% of documented cases (P <0.001), and viral infection was more common in the summer (87.5% in summer vs. 72.3% in other seasons, P <0.001). Outbreaks most often started in Nîmes and moved eastward toward Marseille at a ~9 km/day speed, and these dynamics significantly correlated with the selected temperature, especially during

summer outbreaks; for example, the incidence of enterovirus-driven outbreaks was highly correlated with temperature with correlation coefficients of 0.64 in Nîmes and 0.72 in Marseille, and its occurrence in Marseille lagged behind that in Nîmes by 1-2 weeks.

Conclusion Tracing CAM outbreak dynamics during this retrospective investigation in southern France yielded a speed of displacement that correlated with the variation in temperature between both cities, and these results provide clues for the next occurrence of undocumented outbreaks.

Introduction

Community-acquired infectious meningitis (CAM), claiming more than 1.3 million patients every year worldwide, has a 40% lethality rate partially depending on the causative pathogen.[1-3] RNA viruses, chiefly Enterovirus, are the most frequently documented pathogens and are the most responsible for benign meningitis, but they rarely cause disability and life-threatening cases following progression of the infection to encephalitis. [3] DNA viruses are mainly responsible for disability and life-threatening infections. [4, 5] of bacterial meningitis, causing more than 50% of annual deaths from all-cause meningitis (290,000) and leaving one in five people who recover with chronic neurological disorder.[6] However, 40-60% of CNS-infecting cases had an unknown etiology.[7] While CMA may evolve as sporadic cases, CAM outbreaks are well described for specific pathogens over a well-defined period of time.[3, 8–11] In Europe, enterovirus outbreaks are recorded annually in summer,[3, 12] causing 4,537 meningitis cases in 15 European countries in 2018 and primarily infecting young people,[3] while herpes encephalitis incidence is estimated at 2-4 cases/1,000,000 people worldwide, mainly infecting older people aged > 50 years.[5] Bacterial outbreaks are usually associated with *Haemophilus influenzae*, *Streptococcus pneumoniae*, and *Neisseria meningitidis* infections, causing 16 million cases between 1990 and 2013, with a high incidence in Sub-Saharan Africa.[8, 13] Sporadic cases of infection with other bacteria with low infectious prevalence can be associated with CAM, depending on the region and population.[8]

Here, investigating a large retrospective series of data from CAM syndrome patients diagnosed in two university hospitals in southern France provided a unique opportunity to describe the temporal and spatial dynamics of CAM syndrome outbreaks in this region, yielding a previously unreported finding of the climate-driven eastward movement of CAM outbreaks in which documented cases masked undocumented ones, and these results provide some analogical clues for the next occurrence of undocumented outbreaks.

Patients and methods

Study design

This retrospective study (December 2014 to December 2019) collected only anonymous data issued from routinely diagnosed cases originating from the University Hospital Institute (IHU) Méditerranée Infection Laboratory, Marseille, and the Department of Microbiology and Hospital Hygiene, Nîmes, located approximately 120 km apart in southern France. This study was retrospective and anonymous and required no specific intervention for any patient and no specific clinical sample. Accordingly, this study was approved by the Ethics Committee of the Institut Médeiterranée Infection under numbers 2021-004 (Ethics Committee of the IHU Méditerranée Infection, Marseille) and 21.03.11 (Interface Recherche Bioéthique Institutional Review Board" Ethics Committee, CHU, Nîmes).

Data selection and setting

Annually, approximately 3,500 CSF samples from Assistance Publique-Hôpitaux de Marseille (APHM) were received at the POC laboratory of the IHU Méditerranée Infection at Marseille, and between 900 and 1,500 CSF samples were investigated at the Department of Microbiology and Hospital Hygiene at Nîmes University Hospital. Only CSF samples from patients clinically suspected of community-acquired meningitis (infections contracted outside of the hospital and diagnosed within 48 h of admission) were included in this study, and all CSF samples from patients undergoing neurosurgery, transplantation, dialysis, and hospitalization for more than 48 hours were excluded from the final database. Additionally, only the data from patients with complete clinical records, including age, sex, sampling date and final diagnosis, were analyzed (**Appendix 1**).

Routine laboratory diagnosis

CSF specimens routinely submitted to the POC laboratory of the two hospitals were examined for the numeration of leukocytes and red blood cells using the NucleoCounter® NC-3000™ apparatus and NucleoView™ software (ChemoMetec Inc., Allerod, Denmark). In parallel, CSF was incorporated into the FilmArray® ME Panel assay (bioMérieux, Marcy-l'Etoile, France) for the multiplex PCR-based detection of 14 pathogens, as previously described. [14,15] Then, depending on the POC primarily diagnostic test, real-time PCR (RT–PCR) and culture were routinely performed to confirm and complete the diagnosis. For any further molecular diagnosis, nucleic acids were extracted from 200 µL of CSF using the EZ1 DNA Kit and the EZ1 Virus Mini Kit v2.0 (Qiagen, Courtaboeuf, France), and any remaining extracted DNA was stored at -80 °C. Target amplification using 43 cycles of RT–PCR was performed in the LightCycler® 480 thermal cycler (Roche, Meylan, France) using a specific program for each targeted pathogen and incorporated 5 µL of RNA and LC480 Probes MasterMix 2X (Roche) or 5 µL of DNA and Takyon No Roxe Probe MasterMix (Eurogentec, Angers, France) in a 20-µL final reaction volume. CSF culture was systematically performed using both Chocolate agar PolyViteX (bioMérieux) and Columbia agar enriched with 5% sheep blood (bioMérieux) media incubated at 37 °C under 5% CO_2 for five days [16]. In addition, Columbia agar enriched with 5% sheep blood (bioMérieux) was inoculated with CSF and incubated in anaerobic conditions for 10 days at 37 °C to select for anaerobic pathogens. Cultured microorganisms were identified by using matrix-assisted laser desorption/ionization time-of-flight mass spectrometry as previously described.[17] In addition to a routine pathogen panel, arthropod-borne viruses, including West-Nile-Virus, Toscana Virus and Usutu Virus, were systematically investigated every year between May 1st and November 30th, according to the Regional Health Agency Surveillance Program, and outside this period according to the

recommendations of clinicians. (https://www.paca.ars.sante.fr/surveillance-epidemiologique-des-infections-virus).

Outbreak definition

The time series of incidence was first smoothed with a moving average using a 9-week window. Then, to make the data across cities comparable, the smoothed time series were standardized (centering and scaling). An outbreak period was defined when the maximum standardized value of incidence was equal to or greater than 0.5 in each period in at least one of the two cities' series. The outbreak start-point was defined as the date when the standardized value of incidence rose above baseline (0), and the outbreak stop-point was defined as the date when the standardized value of incidence fell below baseline. For cases of complex outbreaks featuring different behaviors (e.g., outbreaks 6 and 8), the period was set manually. Finally, climatic data recovered from the French National Weather Registry (https://www.historique-meteo.net/france/), including temperature, humidity, and wind, were superimposed with epidemiological data to test for any significant correlation with outbreak patterns.

Statistical analyses

The R software version (3.6.1) [18] was used for all the statistical analyses in this work. All statistical tests were two sided, and the type one error rate was set to 0.05. The quantitative variables are reported as the mean ± SD, and qualitative variables are reported as N (%). For two-group comparisons (e.g., across the two hospitals), Mann–Whitney or Student's t tests were used (as appropriate) for quantitative variables, and Chi-squared or Fisher's exact tests were used (as appropriate) for qualitative variables. The test results were first considered

binary (positive or documented/negative or undocumented) and were evaluated according to location (Nîmes, Marseille) and season of the outbreak and patient sex and age. To compare the positivity status using a multivariate analysis (age, sex, ecology, city), a logistic regression model was used, and the adjusted odds ratio (AOR) was reported. Then, the test results were considered within the context of the identified pathogens. Multiple correspondence analysis (MCA) was performed (with the FactoMineR package [19] to explore the relationships of the incidence of specific pathogens with patient age group and sex and the location, season and period of the outbreak. To track the outbreak patterns of documented cases, cases of documented etiologies were placed into three major groups: DNA viruses, RNA viruses and bacteria. The observations obtained with MCA were confirmed by Fisher's exact test when necessary. In a subsequent analysis, to track the outbreak patterns of documented cases, cases of documented etiologies were placed into three major groups: DNA viruses, RNA viruses and bacteria. To better explore the trends and seasonality of the series, data were then managed as time series using the stats and forecast packages in R [20] in particular to define the outbreak periods (as previously mentioned) and to compute the average delay between infections occurring in Nîmes and Marseille regarding the occurrence of outbreaks and temperatures.

Results

General data

Based on the inclusion criteria, 20,779/28,495 (72.9%) of CSF samples (one CSF sample per patient presenting with a meningitis syndrome) investigated between December 2014 and December 2019 in IHU Méditerranée Infection, Marseille (15,246 CSFs) and CHU Nîmes (5,533 CSFs) laboratories were retrospectively included over this 61-month study held

between 1 December 2014 and 31 December 2019. There was a general trend of an increase in the number of documented cases since 2017 in both cities, whereas we observed a difference in the behavior of the two cities regarding the number of undocumented cases, which strongly decreased in 2016 and returned to baseline in 2017 in Nîmes but increased from mid-2018 in Marseille. Overall, males formed a marginal majority (52.77%; 54.3% in Marseille, 48.6% in Nîmes) ($P < 10^{-4}$), while females were the majority specifically among documented cases (49.4% male patients; 54.5% in Marseille and 42.8% in Nîmes) ($P < 10^{-4}$) (**Table 1**). Additionally, the CAM population was younger in Marseille than in Nîmes (mean age: 40.7 ± 27.2 years vs. 47.2 ± 25.6, respectively) ($P < 0.001$) (**Table 1**), but this difference did not result in any detectably significant influence on the fact that the overall prevalence of documented CAM cases significantly decreased with age for each age group when compared to the prevalence of the reference age group of [0-2[years, except in the patients in the age group of 2-16 years, which presented a higher prevalence (adjusted odds ratio, AOR=0.85, p= 0.0461) (**Figure** 1). Logistic regression results showed that the number of documented cases was significantly higher in Nîmes that in Marseille (AOR=2.8, p<0.001), in females than in males (AOR=1.13, p=0.01), and in the 0-2 and 2-16 age groups than in the other age groups (AOR=1.38, 2.18, 3.69, p<0.001). The most exhaustively diagnosed age group was that aged 2-16 years (AOR=0.85, $P = 0.11$) (**Figure 1**). This observation was confirmed by the chi-squared test, highlighting a significant difference in the repartition of ages between total (documented plus nondocumented) CAM cases and documented CAM cases ($P < 0.001$).

General epidemiology

A total of 2,209 (10.63%) patients were documented in both centers after laboratory investigations as reported above; 1,762 (78.8%) patients had viral meningitis, including 17 coinfections with virus/virus (0.8%), 18 coinfections with virus/bacteria (0.8%), one

coinfection with virus/bacteria/bacteria (0.04%) and two coinfections with virus/virus/bacteria (0.09%); 461 patients had bacterial meningitis (20.9%), including nine coinfections with bacteria/bacteria (0.4%), one coinfection with virus/bacteria/bacteria (0.04%) and 18 coinfections with virus/bacteria (0.8%); and six patients had fungal meningitis (0.3%) (**Table 2, Appendix 1**). CAM was caused by a total of 62 different microorganisms, including 49 bacteria (79%), 12 viruses (19%, 7 DNA and 5 RNA viruses) and one fungal pathogen (*Cryptococcus* 2%), each with variable contributions to CAMs (**Figure 2, Table 2**). Viruses were the main causative pathogens, and 12 viral species were identified in 79.8% of documented CSFs, mainly Enteroviruses (797, 36.1%), HSV-1 (395, 17.9%), HSV-2 (205, 9.3%) and VZV (223, 10.1%) (**Figure 2, Table 2**). Dengue and BK viruses, JC virus, Toscana virus and West Nile virus were detected at a very low frequency in the studied population (**Table 2**). *Streptococcus pneumoniae* (129, 5.8%), *Neisseria meningitidis* (59, 2.7%) and *Haemophilus influenzae* (45, 2%) were the most prevalent bacteria, followed by *Escherichia coli* (34, 1.5%), *Cutibacterium acnes* (31, 1.4%), *Staphylococcus epidermidis* (30, 1.4%), and *Streptococcus agalactiae* (25, 1.1%). *Tropheryma whipplei*, *Staphylococcus aureus* and *Listeria monocytogenes* were detected in rare cases. Additionally, only six cases of *Cryptococcus* meningitis were detected, all in immunocompromised patients (**Table 2**).

Relationships were noted between age and pathogen type; Enterovirus was prevalent in patients aged <16 years (59.8%); HHV-6 in patients aged 0-16 and >60 years (71.6%); HSV1, HSV-2 and VZV in patients aged 16-31 and > 60 years (60.2%, 61.5% and 60.5%, respectively); *H. influenzae* in patients aged 0-2 and >60 years (68.9%); *S. pneumoniae* in patients aged > 45 years (67.4%); *N. meningitidis* in patients aged < 30 years (64.4%); and Human parechovirus and *S. agalactiae* in patients aged 0-2 years (97% and 64%, respectively) (**Table 2, Figures 2 & 3**). Moreover, MCA indicated a preferential association between sex and the causative pathogen, as HSV-1 and HSV-2 were mainly documented in

older female patients (73.7% and 57.7% and $p<10^{-13}$ and $p<10^{-4}$, respectively), whereas enterovirus infection was mostly identified among young male patients (55.7%, p=0.12).

Finally, some seasonal infections, as illustrated by MCA investigation and confirmed by statistical analyses for significance, were observed during the study period for some pathogens ($p < 0.001$), including enterovirus and Human parechovirus, which were mainly documented in the summer and fall seasons, and *N. meningitidis*, *S. pneumoniae*, *S. agalactiae*, HSV-1, HSV-2, VZV, and Cytomegalovirus, which were mainly documented in the winter and spring seasons (**Figure 3, Appendix 3**).

Outbreaks

Ten outbreaks as defined by the criteria above, comprising 12,919/20,779 (62.2%) patients and 1,438/2,209 (65.1%) documented cases, were observed over 61 months (**Figure 4A**). There was a trend toward an increasing frequency of outbreak occurrence over these 61 months. Notably, outbreaks mainly started in Nîmes and appeared eastward in Marseille with an average lag of 1-2 weeks (~13 days). This observation was confirmed with the use of time series decomposition (using the decompose function of the stats package in R) and the use of a cross-correlation function performed on the seasonal regions of the decompositions of the Nîmes and Marseille series. Considering that the two cities are 120 km apart, we measured an average eastward displacement speed of ~0.9 km/day (**Figure 4A**).

Each outbreak included 991-1,834 (median, 1,289) patients, of whom only 100-310 (median, 125) were documented; this ratio of 11.3% was significantly higher than the 10.1% ratio found in outside outbreaks (p=0.01) (**Figure 4**). Specifically, outbreak-1 (January to Mid-March 2015) included 100/1,292 (7.7%) documented patients infected by HSV-1 (29%), HSV-2 (8%), VZV (12%), *S. pneumoniae* (9%), *N. meningitidis* (5%) and Enterovirus (8%);

outbreak-2 (April-July 2015) included 141/1,103 (12.8%) documented patients mainly infected by Enterovirus (64.5%), HSV-1 (10.6%) and VZV (9.9%), which affected children aged < 16 years old in 45.4% of cases; outbreak-3 (January-Mid-March 2016) included 102/1,212 (8.4%) documented patients mainly infected by HSV-1 (27.4%), Enterovirus (21.6%), VZV (14.7%), *S. pneumoniae* (8.8%), *N. meningitidis* (4.9%), and HHV-6 (4.9%); outbreak-4 (March-July 2016) included 118/1,321 (8.94%) documented patients, of which 44.1% of infections were caused by Enterovirus; outbreak-5 (March-July 2017) included 205/1,399 (14.6%) documented patients, of which 70% of infections were caused by Enterovirus; outbreak-6 (November 2017-February 2018) included 132/1366 (9.7%) documented patients with infections caused by HSV-1 (27.3%), HSV-2 (10.6%), VZV (11.4%), *S. pneumoniae* (9.1%), Enterovirus (9.1%), HHV-6 (8.3%), *N. meningitidis* (5.3%), and *S. agalactiae* (3.8%); outbreak-7 (May-September 2018) included 310/1,834 (16.9%) documented patients with infections caused by Enterovirus (50.6%), and VZV (10%), HSV-1 (8.4%), and Human parechovirus (7.1%); outbreak-8 (November 2018-February 2019) included 117/1,286 (9.11%) documented patients infected by HSV-1 (31.6%), VZV (8.5%), *S. pneumoniae* (12%), Enterovirus (9.4%), and *H. influenza* (7.7%); and outbreak-9 (May-July 2019) included 137/991 (13.8%) documented patients mainly infected by Enterovirus (42.3%). Finally, outbreak 10 (August-October 2019) was the least documented outbreak, with only 109/1,245 (8.7%) documented patients infected, and >70% of these cases were caused by one of several neurotropic viruses (**Figure 4**). In summary, winter outbreaks 1, 3, 6 and 8 included infections by HSV-1 (28.8%), HSV-2 (7.3%), VZV (11.5%), *S. pneumoniae* (9.7%), HHV-6 (6.2%), *N. meningitidis* (3.9%), *S. agalactiae* (2.2%), and *Tropheryma whipplei* (2%), leaving 91.2% of cases undocumented, while summer outbreaks 2, 4, 5, 7, and 9 included infections by Enterovirus and Human parechovirus (specifically in 2018) and left 86.48% of cases undocumented (**Figure 3, 4**). Incorporating the incidence of documented vs.

undocumented cases, patient age, outbreak period, and season as variables, MCA clustered the occurrence of summer undocumented cases with patients aged < 31 years old and infection with enterovirus and Human parechovirus, and MCA clustered winter undocumented cases with patients aged > 45 years old and infection with *N. meningitidis*, *S. pneumoniae*, HSV, VZV, and Cytomegalovirus (**Figure 3**). Outside outbreak periods, we observed a persistence of infection with HSV-2, HHV-6, and some bacteria, such as *H. influenzae*, and with other nonfrequent bacteria without any detected association with sex (**Appendix 2**). After etiologies were grouped, reanalysis of the data indicated that RNA viruses occurred significantly more frequently in summer, with a rapid and strong increase (61%, $p<10^{-4}$) and with 52.8% of outbreaks occurring in summer. DNA viruses were identified significantly more frequently in the winter/spring season (54%, $p<10^{-4}$), while no specific relationship between bacterial infection and season was observed despite the significant increase in the frequency of bacterial infection that occurred during winter outbreaks (30.5%, $p<10^{-4}$) (**Figure 5**). However, for outbreaks 7, 8 and 9, which occurred at the same time as outbreaks of RNA viruses in summer and autumn 2018 and summer 2019, there was no clear association between patient age and sex. The same pattern of an ~1 week displacement moving in the same direction was observed by superimposing temperature data. The one-week delay in temperature displacement from Nîmes to Marseille was significantly correlated ($P < 0.001$) with the one-week delay in CAM outbreak displacement, whereas no such significant shifting was observed for the variables “humidity” and “wind” (**Figure 4B**).

Discussion

Retrospective investigation of a large series of CAMs in southern France yielded a complex, dynamic epidemiological pattern combining seemingly sporadic cases and clustered cases, later forming a total of ten outbreaks occurring over 61 months of investigation.[3,7] The

investigation covered cases from two university hospitals in cities separated by 120 km but located in the same region, in which CAM cases were investigated using the same laboratory protocols, allowing for the unprecedented observation of CAM outbreak temporal and spatial dynamics, which were characterized in this region by an eastward displacement at an average of ~9 km/day. The same displacement pattern was observed with temperature records and correlated with outbreaks consistently originating in Nîmes before moving to Marseille, and these data provided the basis for an analysis of the influence of atmospheric temperature and geography on the dynamics of CAM outbreaks; temperature was disclosed to be significantly associated with the displacement dynamics of CAM outbreaks.[3, 21, 22]

Whether temperature was just a marker for certain changing biological conditions in populations, pathogens, and vectors or whether it was a direct biological determinant remains uncertain in this study; an unanticipated observation was that eight CAM syndrome outbreaks varied in the proportion of documented cases and nondocumented cases, while two outbreaks overwhelming comprised undocumented cases (**Figure 4**). Documented enterovirus cases were responsible for six seasonal outbreaks among the ten outbreaks observed here and affected young patient populations as previously described.[3, 23–28] Accordingly, in 2018,[3] the unusual persistence of mixed enterovirus and Human parechovirus outbreaks infecting newborns and children until the autumn correlated with a notable + 2 °C increase in autumnal temperature in October/November 2018 compared to seasonal norms.[29] Most intriguing was the observation that DNA viruses also adopted an outbreak pattern, with HSV-1 CAM being observed in elderly male patients in January-March, probably due to the HSV-1 reactivation,[30, 31] which is potentially prompted by vitamin D deficiency related to low sun exposure in winter, especially in people > 70 years old, as previously reported.[32–34]

By superimposing characteristics of undocumented patients with documented ones, MCA shed light on at least two seasonal patterns for such undocumented outbreaks,

stimulating pathways of future research. In summer, undocumented cases closely clustered with the incidence of RNA viruses, whereas in fall and winter, they clustered with that of DNA viruses and bacteria. This trend could clarify that the increase in hospital admission in summer may be due to infection with RNA viruses, and the high admission of older patients in the fall/winter season is probably due to infection with DNA viruses and/or bacterial nonroutinely investigated at the POC laboratories.[14] Indeed, not all causative pathogens were routinely targeted at POC laboratories in this study, including emerging genotypes and arthropod-borne viruses escaping routine detection.[13,34,35] We propose that Enteroviruses and arthropod-borne viruses are two groups of candidate pathogens to be further examined to account for remaining undocumented cases.

Conclusion

This retrospective study shed light on the significant correlation between temperature and the occurrence of CAM outbreaks in southern France, indicating the necessity of developing new laboratory tools for the search for probable RNA viruses responsible for the currently undocumented majority of cases of CAMs in summer/fall in this region. Real-time metagenomics based on pathogen genome detection performed directly from CSF could be part of this new strategy, with the aim of reducing the number of undocumented CAMs.[35]

Statements & Declarations

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Conflict of interest statement

The authors have no conflicts of interest to declare.

Authors' contributions statment

MM: data collection, data cleaning, design of the study, data interpreting, validation and writing of the manuscript. SF: data cleaning, statistical analysis, data interpretation, validation and writing of the manuscript. KQ, BA, SR, DRC and ZC clinical data collection and data interpretation. LJP and DM, design of the study, data interpretation, validation, funding, critically reviewing of the manuscript, coordination, and direction the work. All authors declare that they have read and approved the manuscript.

Ethics statement

This study was approved by the Ethics Committee of the Institut Médeiterranée Infection in Marseille under numbers 2021-004 (Ethics Committee of the IHU Méditerranée Infection,

Marseille) and by Interface Recherche Bioéthique Institutional Review Board" Ethics Committee, CHU, Nîmes, at Nîmes university hospital under number 21.03.11.

Data access statement

Not applicale

Consent to participate

Not applicable.

Consent to publish

Not applicable.

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Figures.

Figure 1: Distribution of meningitis cases according to patient age. **A)** Boxplot of total negative (undocumented) and positive (documented) meningitis cases according to patient age. **B)** Bar plot of meningitis cases and positive diagnoses according to patient age groups.

Figure 2: Comparison of the microbial diversity of community-acquired meningitis. Patient age-dependent pathogen diversity: patients aged [0,2[and [2,16[were mostly diagnosed positive for enterovirus, patient of [16,46[years-old were in majority positive for Enterovirus, HSV-1 and HSV-2, patient of > 46 years were positive for HSV-1, HSV-2, VZV and bacterial infections. The legend shows in the absolute abondance of each pathogen. Pathogens with global abundance less than 1% are gathered in group "Other".
Abbreviation: Evs, Enterovirus; HSV-1, Herpes Simplex Virus 1; HSV-2, Herpes Simplex Virus 2; VZV, Varicella Zoster Virus; HHV-6, Human Herpes Virus 6.

Figure 3: Multiple correspondence analysis of positive/negative (documented/undocumented) cases, pathogens, and season according to the different outbreaks. Infections in Marseille were more closely associated with summer outbreaks and younger patients, whereas infections in Nîmes were more associated with winter outbreaks and older patients, mostly HSV-1, HSV-2, VZV, and *H. influenzae*. There was no notable difference concerning sex.
Figure 4: Synchronisation of outbreaks and weather data between Marseille and Nîmes by time series analysis. **A)** The time series for temperatures, humidity, wind force (in Marignane and Montpellier, two cities close to Marseille and Nimes), undocumented and documented cases are represented for both Marseille (in cyan) and Nimes (in red). Each time series is represented in its real values (thin lines) and smoothed with moving average using a window

size of 9 weeks (thick lines). At the right-hand size of each time series graphic, the associated cross-correlation function plot comparing Nimes and Marseille at different lags is shown. A high correlation at a negative lag should be interpreted as an event occurring before in Nimes and after in Marseille.

B) Time series were first smoothed using moving average with window size 9 weeks. Then, in order to make the cities and different variables more comparable, a standardization was applied (centering and scaling). The ten outbreaks' periods were defined based on undocumented cases (graphic on the top). The standardized and smoothed time series of undocumented cases, documented cases and specifically RNA virus cases are shown in the three graphics (thick red and cyan lines) along with standardized and smoothed temperatures in Nimes and Marseille (red and cyan thin dotted lines). At the right-hand size of each time series graphic, the associated cross-correlation function plot comparing temperature and respectively undocumented, documented and RNA virus (from top to bottom) are shown in red for Nimes and in cyan for Marseille.

Figure 5: Multiple Correspondence Analysis (MCA) of variables seasons, outbreak number, identified pathogens gathered by microorganisms' groups (RNA virus, DNA virus, Bacteria and Fungi), gender and age classes. In summer five outbreaks closely caused by RNA viruses infecting young patients (61%), represented in 52.8% ($p<10^{-4}$) of summer outbreaks. DNA viruses occurred in all seasons in a more equal distribution but accounted for ~54% ($p<10^{-4}$) of both spring and winter outbreaks. Although bacteria were less season-specific, they were nevertheless more frequent in winter (30% of bacteria were detected in winter) and accounted for 30.5% of winter epidemics ($p<10^{-4}$). According to the ACM, RNA viruses are the furthest from the center of the benchmark and are associated with summer. Bacteria and DNA viruses are closer to the center, so less season-specific, but still closer to winter and spring. Mainly

undocumented cases clustered with bacteria and DNA viruses in winter and with all etiologies in summer notably with RNA viruses in majority especially young patients.

*Fisher's Exact Test for Count Data with simulated p value (based on 2000 replicates), (p value = 0.0004998).

Tables.

Table 1: Summary statistics for variables Age and Gender, comparisons according to cities and positive/negative status.

Table 2: Prevalence of pathogens by age classes for period 12/2014-12/2019 (Date from Nîmes and Marseille gathered).

Table 1

	Global tested population				Positive result population				
	TOTAL	Marseille	Nimes	p-value (Nimes vs Marseille)	TOTAL	Marseille	Nimes	p-value (Nimes vs Marseille)	p-value (Global vs Positives)
N	20779 (100%)	15246 (73.4%)	5533 (26.6%)	-	2209	1238	971	-	<0.001
Gender (Men)	10966 (52.77%)	8274 (54.27%)	2692 (48.65%)	<0.001	1091 (49.39%)	675 (54.52%)	416 (42.84%)	<0.001	<0.001
Age	42.46 ± 26.94	40.75 ± 27.22	47.19 ± 25.57	<0.001	31.43 ± 27.56	27.4 ± 2 6.84	36.57 ± 27.62	<0.001	<0.001
Bacteria	-	-	-	-	471 (21.32%)	376 (30.37%)	95 (9.78%)	<0.001	-
DNA Virus	-	-	-	-	934 (42.28%)	296 (23.91%)	638 (65.70%)		
Fungi	-	-	-	-	6 (0.27%)	6 (0.48%)	0 (0%)		
RNA Virus	-	-	-	-	847 (38.34%)	599 (48.38%)	248 (25.54%)		

Table 2

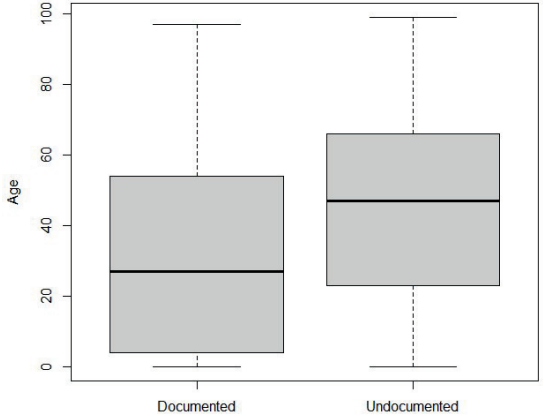
	[0,2[	[2,16[	[16,31[	[31,46[	[46,60[	>60	Total
Enterovirus	291 (36.5%)	186	170	141	5 (0.6%)	4 (0.5%)	797
Herpes Simplex Virus 1	17 (4.3%)	28	99	58	54	139	395
Varicella Zoster Virus	12 (5.4%)	28	37	23	25	98	223
Herpes Simplex Virus 2	6 (2.9%)	6 (2.9%)	67	39 (19%)	28	59	205
Streptococcus pneumoniae	17 (13.2%)	18 (14%)	7 (5.4%)	15	27	45	129
Human Herpesvirus Virus 6	30 (37%)	10	7 (8.6%)	7 (8.6%)	9	18	81
Neisseria meningitidis	12 (20.3%)	9	17	7	8	6 (10.2%)	59
Haemophilus influenzae	11 (24.4%)	2 (4.4%)	6	5	1 (2.2%)	20	45
Escherichia coli	16 (47.1%)	0 (0%)	3 (8.8%)	4	1 (2.9%)	10	34
Human parechovirus	33 (97.1%)	1 (2.9%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	34
Cutibacterium acnes	1 (3.2%)	2 (6.5%)	9 (29%)	3 (9.7%)	6	10	31
Staphylococcus epidermidis	6 (20%)	1 (3.3%)	5	1 (3.3%)	10	7 (23.3%)	30
Streptococcus agalactiae	16 (64%)	0 (0%)	1 (4%)	1 (4%)	4 (16%)	3 (12%)	25
Tropheryma whipplei	0 (0%)	0 (0%)	2 (10%)	2 (10%)	8 (40%)	8 (40%)	20
Staphylococcus aureus	3 (21.4%)	1 (7.1%)	0 (0%)	1 (7.1%)	2	7 (50%)	14
JC Virus	0 (0%)	0 (0%)	0 (0%)	0 (0%)	4	8 (66.7%)	12

	[0,2[	[2,16[	[16,31[	[31,46[	[46,60[	>60	Total
Cytomegalovirus	2 (18.2%)	1 (9.1%)	2	2	2	2 (18.2%)	11
Dengue virus	0 (0%)	1 (10%)	4 (40%)	2 (20%)	3 (30%)	0 (0%)	10
Staphylococcus hominis	2 (22.2%)	0 (0%)	1	2	1	3 (33.3%)	9
BK Virus	0 (0%)	1	0 (0%)	1	2	3 (42.9%)	7
Listeria monocytogenes	0 (0%)	0 (0%)	0 (0%)	1	2	4 (57.1%)	7
Cryptococcus meoformans/gattii	0 (0%)	0 (0%)	2	2	1	1 (16.7%)	6
Klebsiella pneumoniae	0 (0%)	0 (0%)	0 (0%)	1 (20%)	2 (40%)	2 (40%)	5
Staphylococcus capitis	2 (40%)	0 (0%)	1 (20%)	0 (0%)	2 (40%)	0 (0%)	5
Toscana virus	0 (0%)	0 (0%)	2 (40%)	0 (0%)	2 (40%)	1 (20%)	5
Treponema pallidum	0 (0%)	0 (0%)	0 (0%)	0 (0%)	5 (100%)	0 (0%)	5
Borrelia sp.	0 (0%)	1 (25%)	0 (0%)	1 (25%)	0 (0%)	2 (50%)	4
Streptococcus pyogenes	0 (0%)	3 (75%)	0 (0%)	0 (0%)	1 (25%)	0 (0%)	4
Enterococcus faecalis	0 (0%)	1	0 (0%)	0 (0%)	2	0 (0%)	3
Pseudomonas aeruginosa	1 (33.3%)	1	0 (0%)	0 (0%)	0 (0%)	1 (33.3%)	3
Staphylococcus haemolyticus	0 (0%)	0 (0%)	0 (0%)	1	1	1 (33.3%)	3
Streptococcus oralis	1 (33.3%)	1	0 (0%)	1	0 (0%)	0 (0%)	3

	[0,2[	[2,16[	[16,31[	[31,46[	[46,60[	>60	Total
Acinetobacter baumannii	0 (0%)	0 (0%)	1 (50%)	1 (50%)	0 (0%)	0 (0%)	2
Enterobacter cloacae	0 (0%)	0 (0%)	1 (50%)	0 (0%)	1 (50%)	0 (0%)	2
Propionibacterium avidum	0 (0%)	0 (0%)	0 (0%)	1 (50%)	0 (0%)	1 (50%)	2
Ureaplasma urealyticum	2 (100%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	2
Acinetobacter lwoffii	0 (0%)	0 (0%)	0 (0%)	0 (0%)	1 (100%)	0 (0%)	1
Acinetobacter radioresistens	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	1 (100%)	1
Bacillus fragilis	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	1 (100%)	1
Bacillus megaterium	0 (0%)	0 (0%)	0 (0%)	1 (100%)	0 (0%)	0 (0%)	1
Bacillus simplex	0 (0%)	0 (0%)	0 (0%)	0 (0%)	1 (100%)	0 (0%)	1
Chlamydophila pneumoniae	0 (0%)	0 (0%)	1 (100%)	0 (0%)	0 (0%)	0 (0%)	1
Citrobacter freundii	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	1 (100%)	1
Citrobacter koseri	1 (100%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	1
Kocuria rhizophila	0 (0%)	0 (0%)	1 (100%)	0 (0%)	0 (0%)	0 (0%)	1
Mycobacterium tuberculosis	0 (0%)	0 (0%)	0 (0%)	0 (0%)	1 (100%)	0 (0%)	1
Pantoea sp.	0 (0%)	0 (0%)	0 (0%)	0 (0%)	1 (100%)	0 (0%)	1
Parvimonas micra	0 (0%)	0 (0%)	0 (0%)	0 (0%)	1 (100%)	0 (0%)	1

	[0,2[	[2,16[	[16,31[	[31,46[	[46,60[	>60	Total
Pasteurella multocida	0 (0%)	0 (0%)	1 (100%)	0 (0%)	0 (0%)	0 (0%)	1
Proteus mirabilis	0 (0%)	0 (0%)	0 (0%)	1 (100%)	0 (0%)	0 (0%)	1
Proteus vulgaris	0 (0%)	0 (0%)	0 (0%)	0 (0%)	1 (100%)	0 (0%)	1
Roseomonas sp	0 (0%)	0 (0%)	0 (0%)	0 (0%)	1 (100%)	0 (0%)	1
Streptococcus anginosus	0 (0%)	0 (0%)	0 (0%)	0 (0%)	1 (100%)	0 (0%)	1
Staphylococcus intermedius	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	1 (100%)	1
Staphylococcus lugdunensis	0 (0%)	0 (0%)	0 (0%)	0 (0%)	1 (100%)	0 (0%)	1
Serratia marcescens	0 (0%)	0 (0%)	0 (0%)	0 (0%)	1 (100%)	0 (0%)	1
Streptococcus mitis	1 (100%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	1
Streptococcus parasanguinis	0 (0%)	0 (0%)	1 (100%)	0 (0%)	0 (0%)	0 (0%)	1
Staphylococcus pasteuri	0 (0%)	0 (0%)	0 (0%)	1 (100%)	0 (0%)	0 (0%)	1
Streptococcus salivarius	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	1 (100%)	1
Staphylococcus warneri	0 (0%)	0 (0%)	0 (0%)	1 (100%)	0 (0%)	0 (0%)	1
Virus West Nile	0 (0%)	0 (0%)	0 (0%)	1 (100%)	0 (0%)	0 (0%)	1
Total	483 (21.4%)	302	448	328	229	468	2,258

A)



B)

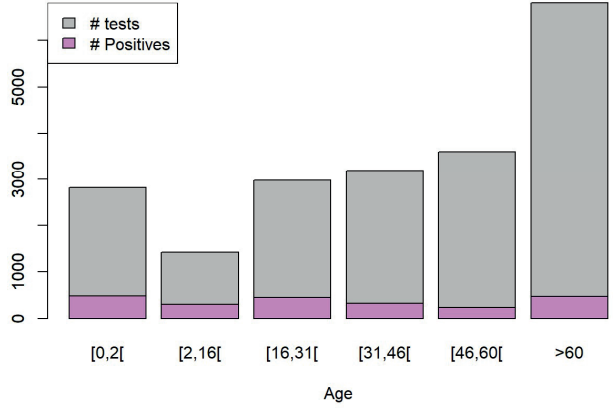


Figure 2

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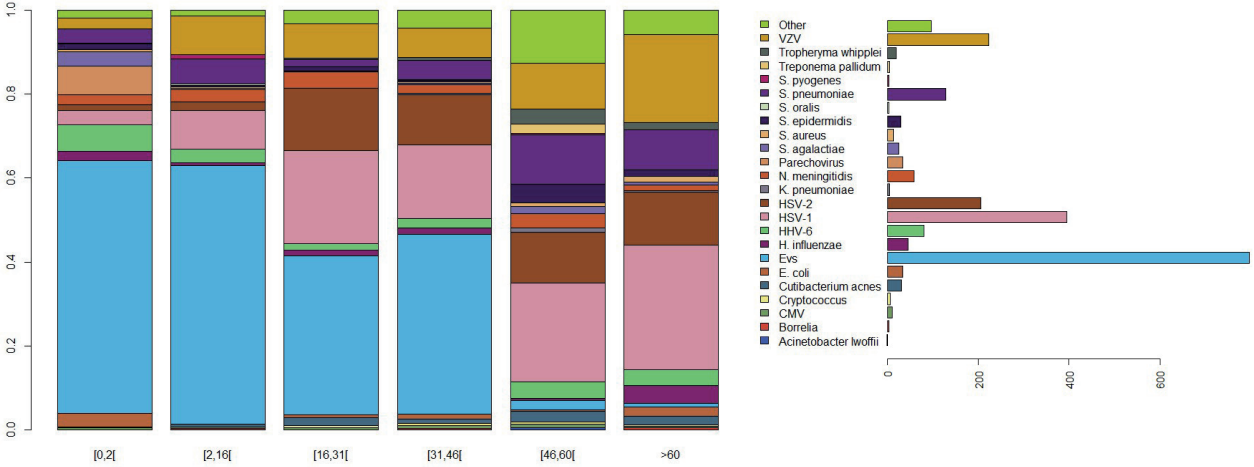

Figure 3

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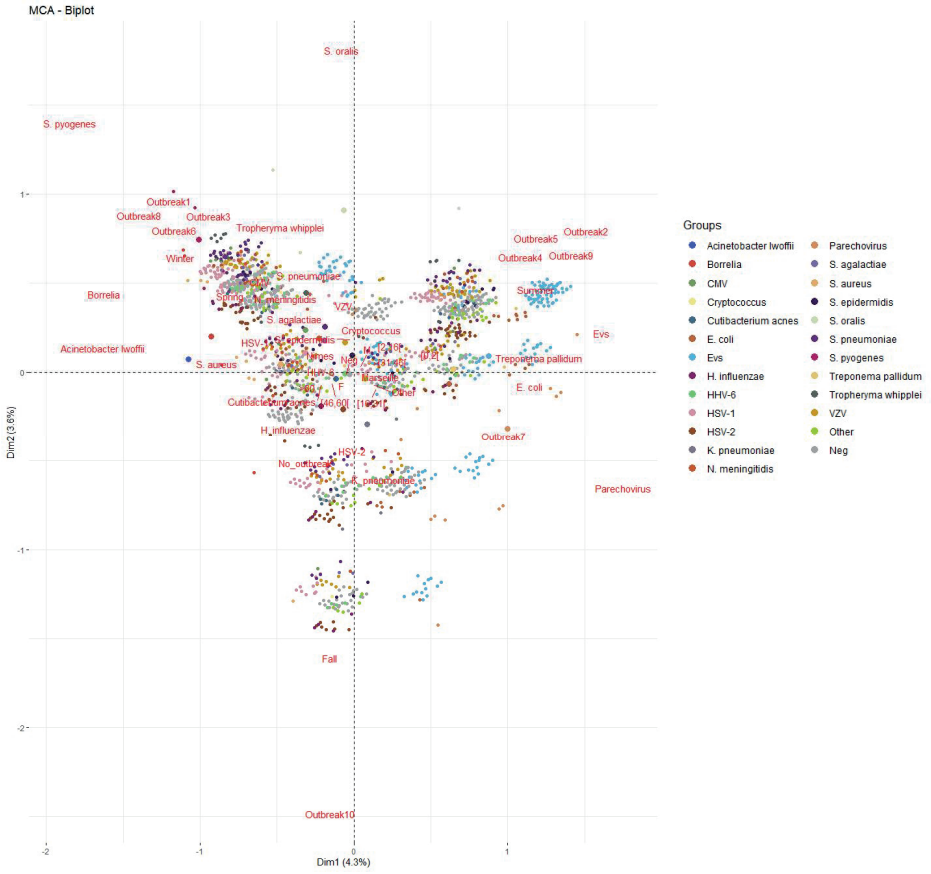

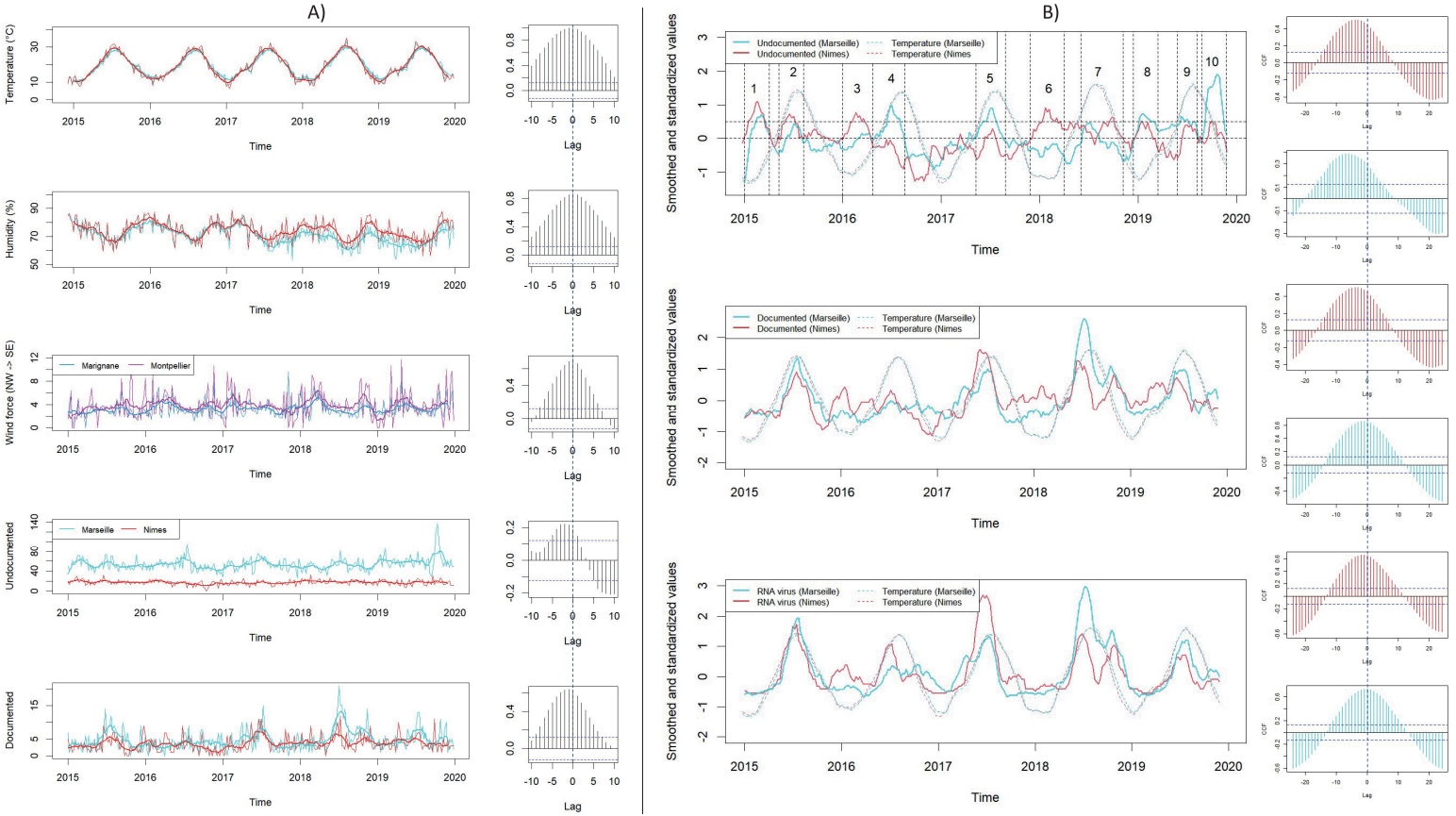

Figure 5

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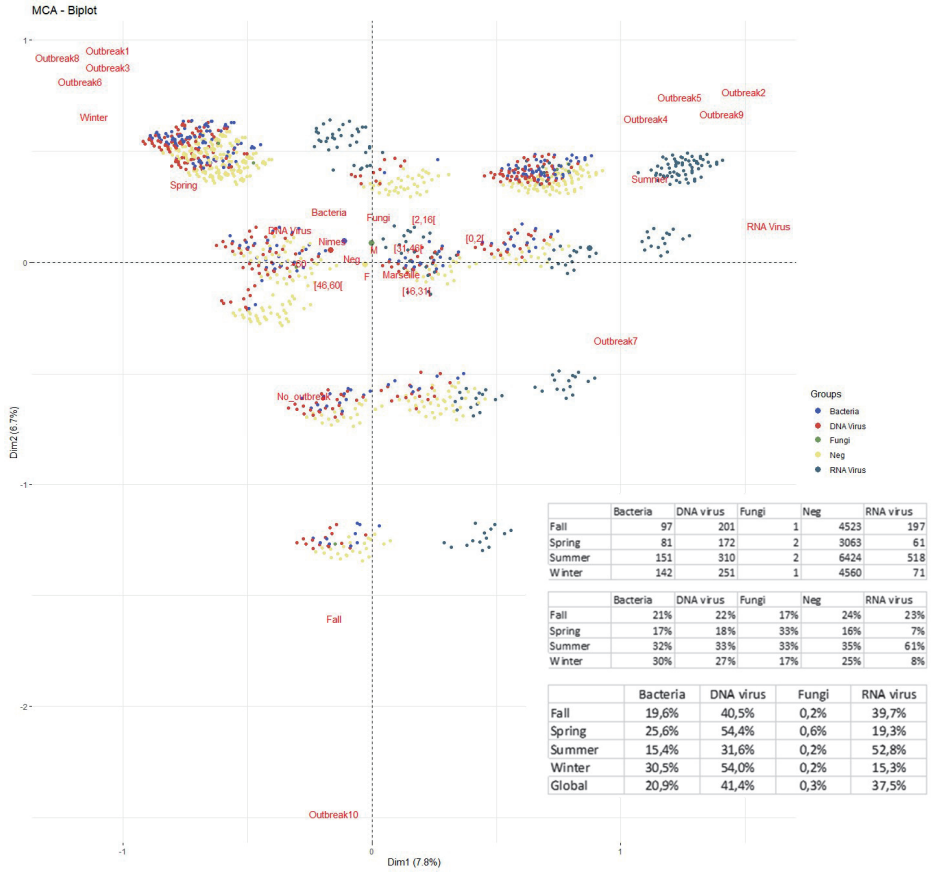

	Bacteria	DNA virus	Fungi	Neg	RNA virus
Fall	97	201	1	4523	197
Spring	81	172	2	3063	61
Summer	151	310	2	6424	518
Winter	142	251	1	4560	71

	Bacteria	DNA virus	Fungi	Neg	RNA virus
Fall	21%	22%	17%	24%	23%
Spring	17%	18%	33%	16%	7%
Summer	32%	33%	33%	35%	61%
Winter	30%	27%	17%	25%	8%

	Bacteria	DNA virus	Fungi	RNA virus
Fall	19,6%	40,5%	0,2%	39,7%
Spring	25,6%	54,4%	0,6%	19,3%
Summer	15,4%	31,6%	0,2%	52,8%
Winter	30,5%	54,0%	0,2%	15,3%
Global	20,9%	41,4%	0,3%	37,5%

STROBE Statement—Checklist of items that should be included in reports of ***cohort studies***

	Item No	Recommendation	Page No
Title and abstract	1	(*a*) Indicate the study's design with a commonly used term in the title or the abstract (*b*) Provide in the abstract an informative and balanced summary of what was done and what was found	1-3
Introduction			
Background/rationale	2	Explain the scientific background and rationale for the investigation being reported	4
Objectives	3	State specific objectives, including any prespecified hypotheses	4
Methods			
Study design	4	Present key elements of study design early in the paper	5
Setting	5	Describe the setting, locations, and relevant dates, including periods of recruitment, exposure, follow-up, and data collection	5
Participants	6	(*a*) Give the eligibility criteria, and the sources and methods of selection of participants. Describe methods of follow-up (*b*) For matched studies, give matching criteria and number of exposed and unexposed	6
Variables	7	Clearly define all outcomes, exposures, predictors, potential confounders, and effect modifiers. Give diagnostic criteria, if applicable	7-8
Data sources/ measurement	8*	For each variable of interest, give sources of data and details of methods of assessment (measurement). Describe comparability of assessment methods if there is more than one group	7-8
Bias	9	Describe any efforts to address potential sources of bias	14-15
Study size	10	Explain how the study size was arrived at	5
Quantitative variables	11	Explain how quantitative variables were handled in the analyses. If applicable, describe which groupings were chosen and why	7-8
Statistical methods	12	(*a*) Describe all statistical methods, including those used to control for confounding (*b*) Describe any methods used to examine subgroups and interactions (*c*) Explain how missing data were addressed (*d*) If applicable, explain how loss to follow-up was addressed (*e*) Describe any sensitivity analyses	7-8
Results			
Participants	13*	(a) Report numbers of individuals at each stage of study—eg numbers potentially eligible, examined for eligibility, confirmed eligible, included in the study, completing follow-up, and analysed (b) Give reasons for non-participation at each stage (c) Consider use of a flow diagram	8
Descriptive data	14*	(a) Give characteristics of study participants (eg demographic, clinical, social) and information on exposures and potential confounders (b) Indicate number of participants with missing data for each variable of interest (c) Summarise follow-up time (eg, average and total amount)	8-9
Outcome data	15*	Report numbers of outcome events or summary measures over time	9-11

Main results	16	(*a*) Give unadjusted estimates and, if applicable, confounder-adjusted estimates and their precision (eg, 95% confidence interval). Make clear which confounders were adjusted for and why they were included (*b*) Report category boundaries when continuous variables were categorized (*c*) If relevant, consider translating estimates of relative risk into absolute risk for a meaningful time period	9-12
Other analyses	17	Report other analyses done—eg analyses of subgroups and interactions, and sensitivity analyses	11-13
Discussion			
Key results	18	Summarise key results with reference to study objectives	13
Limitations	19	Discuss limitations of the study, taking into account sources of potential bias or imprecision. Discuss both direction and magnitude of any potential bias	14
Interpretation	20	Give a cautious overall interpretation of results considering objectives, limitations, multiplicity of analyses, results from similar studies, and other relevant evidence	14-15
Generalisability	21	Discuss the generalisability (external validity) of the study results	13-15
Other information			
Funding	22	Give the source of funding and the role of the funders for the present study and, if applicable, for the original study on which the present article is based	16

*****Give information separately for exposed and unexposed groups.

Note: An Explanation and Elaboration article discusses each checklist item and gives methodological background and published examples of transparent reporting. The STROBE checklist is best used in conjunction with this article (freely available on the Web sites of PLoS Medicine at http://www.plosmedicine.org/, Annals of Internal Medicine at http://www.annals.org/, and Epidemiology at http://www.epidem.com/). Information on the STROBE Initiative is available at http://www.strobe-statement.org.

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Chapitre III : Diagnostic étendu par métagénomique des méningites communautaires à Entérovirus.

Préambule

Les Entérovirus sont les virus les plus fréquemment identifiés chez les patients enfants et adultes présentant un syndrome méningé communautaire (8,12,59–63). Ces virus à ARN non encapsidés de la famille des *Picornaviridae* ont un génome variant entre 7.000 et 8.000 nucléotides (60,64) et parmi au moins 300 sérotypes répartis entre 15 espèces différentes, 100 sont pathogènes pour l'Homme (11,60,63,65). Le pronostic d'une méningite à Entérovirus dépend essentiellement du génotype de virus causatif (11): En effet, les méningites à Entérovirus sont toujours bénignes à l'exception de celles causées par Entérovirus A 71 et Enterovirus D68, associés à des cas sévères parfois mortels de méningoencéphalite, également associés à des paralysies séquellaires (7,11,60,66,67). Il est donc d'intérêt médical pour la décision d'hospitalisation du patient, de diagnostiquer par génotypage les Entérovirus détectés dans le LCR, dans le temps du soin. Le diagnostic des méningites à Entérovirus est basé sur des PCR multiplexées reflétant une approche syndromique, commercialement disponibles (25,28–30); complétées par une amplification-séquençage partiel de gène VP1 pour le génotypage (6,63,68); compliqué par la diversité génomique liée aux recombinaisons génétiques des Entérovirus, explorée par le séquençage d'un long fragment de génome (7,11,69).

Dans le travail exposé dans ce chapitre, nous avons proposé deux pistes expérimentales pour le diagnostic et le génotypage des Entérovirus à partir de LCR. D'une part, basé sur une publication dans le journal Nature (70), nous avons mis en place un protocole d'enrichissement du génome d'Entérovirus dans le LCR en utilisant 46 amorces chevauchantes qui couvrent tout le génome, dont le produit d'amplification a été engagé directement dans la préparation de la librairie Illumina et séquencé dans des séquenceurs Illumina Miseq et iSeq. Plus de 40% de génome d'un Echovirus 9 ont été identifiés par analyse bioinformatique alors que moins de 7% de génome analysés par séquençage partiel de gène VP1 ont conduit à une identification erronée d'Echovirus 7.

Un deuxième protocole sans enrichissement et sans amplification préalable nous a permis d'identifier et caractériser Echovirus sérotype 12 dans un cas de méningite communautaire chez un nouveau-né. Ce deuxième protocole comporte une extraction d'ARN par billes magnétiques, suivi d'une cascade de traitement par des DNases et de purifications. Une étape de rétrotranscription et la synthèse de l'ADN double brin suivant le protocole Klenow a été réalisée. L'ARN généré a été utilisé pour préparer la librairie Illumina et séquencé dans un Illumina iSeq. Dans ces conditions, plus de 80% de génome d'Echovirus 12 ont été détectés directement à partir du LCR, alors que le séquençage de 300 nucléotides du gène VP1 en routine avait identifié Echovirus 9. Dans ce travail, nous avons estimé un coût par nucléotide de 0,3 € pour le séquençage Sanger et de 0,03 € pour le séquençage NGS avec la technologie Illumina. Dans l'ensemble, cette étude illustre

l'intérêt du mNGS dans le cadre du diagnostic de routine de la méningite en temps et en coût.

Article 3

Direct next-generation sequencing diagnosis of echovirus 9 meningitis, France.

Madjid Morsli [1,2]. Jean-Jacques Vincent[1,3]. Laurine Milliere[1,3]. Philippe Colson[1,2]. Michel Drancourt[1,2,3]

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BRIEF REPORT

Direct next-generation sequencing diagnosis of echovirus 9 meningitis, France

Madjid Morsli[1,2] · Jean-Jacques Vincent[1,3] · Laurine Milliere[1,3] · Philippe Colson[1,2] · Michel Drancourt[1,2,3]

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Abstract
The prognosis of central nervous system infections caused by enteroviruses partially depends on the viral genotype, which is not provided by current point-of-care diagnostic methods. In this study, next-generation sequencing identified an echovirus 9 directly from the cerebrospinal fluid of a patient presenting with meningitis.

Keywords Enterovirus · meningitis · Next-generation sequencing · Genotype · Point-of-care diagnosis · Echovirus

Introduction

Enteroviruses are non-enveloped viruses with 7200–8500 nucleotide-long single-stranded positive RNA genomes that comprise a genus within the *Picornaviridae* family [1]. Enterovirus diversity is considerable, as more than 300 genotypes of human non-polio enteroviruses have been identified, and they have been divided into four subgroups A–D [1]. Non-polio enteroviruses are common agents worldwide of neurological infections that are usually asymptomatic or mild and spontaneously resolving [2]. Nevertheless, some enterovirus genotypes such as enterovirus-A71 or enterovirus-D68 are associated with neurovirulence and fatalities [3, 4]. Knowledge of the viral genotype is therefore of potential clinical concern, and the prevalence of genotypes of enteroviruses from patients presenting with meningitis and encephalitis is currently not broadly characterised [5]. Current molecular diagnostic tests of enterovirus neurological infections based on simplex or multiplex real-time reverse transcription (RT)-PCR assays [6] do not reveal the viral genotype, which is most usually determined by analysing a fragment of the VP1 capsid-encoding gene [7]. Next-generation sequencing is a promising approach for enterovirus genotyping that previously most often required PCR amplification [8].

Here, we implemented a protocol that used spiked primer-based enrichment followed by next-generation sequencing (NGS) for the detection and identification of enteroviruses directly from the cerebrospinal fluid (CSF) of a meningitis patient.

Materials and methods

A 25-year-old woman was admitted at the emergency department for a 3-day history of febrile (39 °C) headache, photophobia, jet vomiting, and neck stiffness. CSF analysis showed a leukocyte count of 100 cells/mm^3 with 100% polynuclear cells, proteins at 0.39 g/L, and glucose at 3.52 mmol/L. Microscopic examination after Gram staining was negative. Enterovirus diagnosis was obtained at the point-of-care (POC) laboratory using the Biofire FilmArray Meningitis/Encephalitis panel (Biomérieux, Marcy-l 'Etoile, France) [9]. At the same time, enterovirus identification was performed using whole genome sequencing after adapting previously described protocol that used a panel of short PCR primers for targeted-sequence enrichment prior to NGS [10]. A total of 78 complete enterovirus genomes were downloaded from the GenBank database (using the keyword "echovirus"), aligned using the Clustal Omega Multiple Sequence Alignment online tool (https://www.ebi.ac.uk/Tools/msa/clustalo/) and manually partitioned into ≈ 500 nucleotide-long fragments overlapping by ≈ 100–250 nucleotides (Fig. 1). Then, 46 primers comprising 13 nucleotides (online

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Published online: 11 March 2021

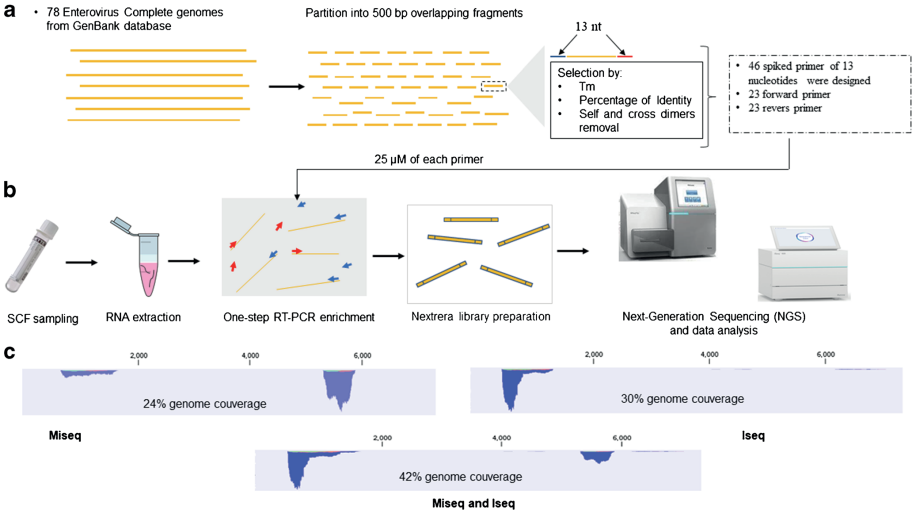

Fig. 1 Next-generation sequencing direct diagnosis of the echovirus 9 meningitis workflow. The total duration of the handling procedure was less than 48 h including different steps. **a** Spiked primer enrichment design. A total of 78 complete enterovirus genomes collected from the GenBank database were partitioned into 500-bp-long fragments after the alignment. A total of 46 13-nucleotide forward and reverse spiked primers were designed in the conserved regions at either ends of fragments. The primer selection was performed according to the Tm, identity percentage and self or cross dimer removals. **b** CSF sampling and total RNA EZ1 extraction followed by one-step RT-PCR amplification containing 25 µM of each primer and 10 µL of genomic RNA, then the amplification product was sequenced by next-generation sequencing (NGS) using iSeq 100 and MiSeq instrument after the library preparation. **c** The NGS was analyzed using the CLC Genomics Workbench, version 7.5.0 (Qiagen), identifying an echovirus E9 genome strain: (LC321988.1)

Appendix 1) were designed to target the extremities of each fragment and checked using the Multiple Primer Analyzer online software (https://www.thermofisher.com/). RT-PCR was performed on an Applied Biosystem thermocycler (Foster City, CA, USA) with the SuperScript One-Step RT-PCR System (Invitrogen, Cergy-Pontoise, Germany) in a 50-µL volume containing 10 µL of total RNA extracted from 200 µL of CSF with the EZ1 Virus Mini Kit v2.0 (Qiagen, Courtaboeuf, France) (Fig. 1). Then, 1 ng of DNA was used for preparation of the NGS library (online Appendix 2). NGS was performed using the Illumina Nextera XT paired-end protocol (Illumina, San Diego, USA), as previously described [11], on iSeq 100 or MiSeq instruments (Illumina) and sequences were analysed with the CLC Genomics Workbench software version 7.5.0 (Qiagen).

Results and discussion

In the absence of enrichment, NGS on MiSeq generated 1,100,212 reads but only 32 (0.003%) could be mapped onto the Echovirus 9 genome GenBank accession no. LC321988.1, which was the best BLASTn hit from the NCBI GenBank nucleotide sequence database. With enrichment, NGS performed on MiSeq generated 1,094,034 reads of which 1040 (0.10%) could be mapped on genome LC321988.1, and NGS on iSeq generated 161,156 reads of which 2172 (1.35%) could be mapped on this genome. Thus, NGS read sets were enriched in enterovirus sequences by between 33 and 463 times, respectively. Mapping all NGS reads obtained post-enrichment on the Echovirus 9 genome showed that 2983 reads matched and allowed generating five contigs covering 3.098 non-contiguous nucleotides of the LC321988.1 genome, corresponding to ≈ 42% of the total genome length (Fig. 1). This represented a 4.6-fold increase in achieved genome coverage compared to without enrichment (679 nucleotides) (accession no. LR877187).

Here, direct whole genome sequencing identified a CSF pathogen as echovirus 9, an identification which was not achieved by parallel multiplex PCR which gave identification at the family level only (enterovirus). Moreover, use of spiked primer-based enrichment improved the depth and coverage of an enterovirus genome directly retrieved from CSF [9]. Only one partial genome was, however, obtained, which may be at

least partly related to the low viral load in the CSF, and/or to the deterioration of the viral RNA genome. Echovirus 9 (formerly Coxsackie A23) is a predominant enterovirus type and among the enteroviruses the most commonly identified in meningitis cases [5]. However, the present diagnosis strategy approach has the potential of identifying emerging and new genotypes. Genome enrichment based on multiplex PCR allows the detection not only of common pathogens but also of uncommon ones whereas conventional mltiplex PCR is limited only to common pathogens [10]. In addition, the implementation of NGS-based genome sequencing in a clinical microbiology laboratory for routine diagnosis will provide significantly more precise information on the genotype than gene-targeting Sanger sequencing, and will expand the database of genomes associated with various clinical presentations, allowing for better insight into associations between genotypes and clinical outcomes. Consequently, such an enrichment-improved whole genome NGS strategy will henceforth be routinely implemented in our POC laboratories, for the accurate diagnosis of enterovirus meningitis and encephalitis.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s10096-021-04205-6.

Code availability Not applicable.

Author contribution MM contributed to experimental design, realization of the work, data analysis, interpretation, and writing. JJV and LM collected samples and clinical information. PC and MD contributed to critically reviewing the manuscript and data interpretation. MD coordinated and directed the work.

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Data Availability GenBank accession nos. LR815569.1, LR815570.1, LR815571.1, LR815572.1, LR815573.1.

Declarations

Ethical Approval All data were generated as part of routine work at the Assistance Publique-Hôpitaux de Marseille (Marseille University hospitals), and this study is the result of routine clinical management. No specific clinical sampling has been done in this study.

Consent to participate Not applicable.

Conflict of interest The authors declare that they have no conflicts of interest.

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Article 4

Direct Diagnosis of Echovirus 12 Meningitis Using Metagenomic Next Generation Sequencing.

Madjid Morsli [1,2], Christine Zandotti [1], Aurelie Morand [3], Philippe Colson [1,2] and Michel Drancourt [1,2,*]

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MDPI

Brief Report

Direct Diagnosis of Echovirus 12 Meningitis Using Metagenomic Next Generation Sequencing

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Abstract: The current point-of-care diagnosis of enterovirus meningitis does not identify the viral genotype, which is prognostic. In this case report, more than 81% of an *Echovirus* 12 genome were detected and identified by metagenomic next-generation sequencing, directly from the cerebrospinal fluid collected in a 6-month-old child with meningeal syndrome and meningitis: introducing *Echovirus* 12 as an etiological agent of acute meningitis in the pediatric population.

Keywords: enterovirus meningitis; whole genome sequencing; metagenomic next-generation sequencing; *Echovirus* 12; cerebrospinal fluid

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1. Introduction

The highly diverse viral genus *Enterovirus* encompasses more than 300 genotypes [1]. Some *Enterovirus* members are responsible for central nervous system (CNS) infections, which clinical and epidemiological characteristics and prognosis, vary according to the precisely identified enterovirus, and some genotypes have been associated with a particular clinical severity and mortality [2]. The *Enterovirus* genotype is not routinely determined by molecular diagnosis assays detecting enterovirus RNA at the core and point-of-care (POC) laboratories [3–6]. *Echovirus* strains belonging to *Enterovirus B* species preferentially infect infants and young children [1,4] and are frequently involved in aseptic meningitis and encephalitis. Indeed, *Enterovirus* genotyping is most commonly based on partial VP1 gene Sanger sequencing using a generic standard protocol which is not commonly applied during the time of care [7].

In order to challenge *Enterovirus* genotyping at the POC laboratory, we herein developed a unique protocol to diagnose and genotype *Enterovirus* CNS infection directly from the cerebrospinal fluid (CSF) using metagenomic Next-Generation Sequencing (mNGS). This diagnosis approach is here illustrated by the diagnosis of *Echovirus* 12 meningitis in a child, a rarely reported situation in such setting [8,9].

2. Case Presentation and Methods

A 6-month-old girl born from twin pregnancy was admitted at the emergency department with fever (37.7 °C), cough and meningeal syndrome. She had an history of Respiratory Syncytial Virus bronchiolitis that led to hospitalization one month prior to meningitis. At the admission, CSF analysis after lumbar puncture showed a leukocyte count of 1 cell/mm^3, protein at 0.14 g/L and glucose at 3.41 mmol/L. Microscopic analysis after Gram staining was negative. Investigation of the CSF at the POC laboratory using the Biofire FilmArray Meningitis/Encephalitis panel (bioMérieux, Marcy-l, Etoile, France) [6] was positive for *Enterovirus*.

In parallel, total RNA was manually extracted from 200 µL of CSF, following an in-house developed protocol, using QIAamp Viral RNA Mini Kit solutions (Qiagen, Hilden, Germany) for lysing, and washing steps, then total RNA was then purified using RNA specific magnetic Dynabeads (Life technology, Oslo, Norway). Briefly, 200 µL of CSF were incubated with 50 µL proteinase K (Qiagen) for 5 min at room temperature, then 300 µL AVL lysis buffer (Qiagen) were added and incubated for 15 min at room temperature. A 150-µL volume absolute ethanol (99%) were added to the lysis mix, 50-µL Dynabeads (40 mg/µL) were added and incubated for 15 min at room temperature. The Dynadeads/RNA complex washed twice with 850 µL AV1 solution (Qiagen), then two times with 450 µL AV2 solution (Qiagen) in the presence of 70% ethanol. After the second wash, Dynabeads were dried for 15 min at room temperature and eluted in a 60 µL-volume, then incubated for 3 min at 70 °C, followed by magnetic separation. Finally, 3 µL of RNaseOUT™ Recombinant Ribonuclease Inhibitor (Invitrogen, Illkirch, France were added to the purified RNA was and stored at −70 °C.

A 40-µL volume of total RNA was treated with ezDNase (Invitrogen, Illkirch, France) and concentrated with (Zymo Research, Irvine, CA, USA) kit, then eluted in 20 µL sterile water (Figure 1). The complementary DNA (cDNA) synthesis was performed using TaqMan kit according to the manufacturer protocol (Applied Biosystem, Foster City, CA, USA) in 50µL containing 19.25 µL eluted RNA, then 20 µL of the cDNA were used as a matrix for double strand synthesis, using 3 units of DNA Polymerase I, Large (Klenow) Fragment (BioLabs) in a 30 µL-volume. Double stranded DNA purified with Agencourt® AMPure beads (Invitrogen) and eluted in 17 µL of 1x-sterile Tris-EDTA solution. Finally, 1 ng of cDNA was used for metagenomics Next-Generation Sequencing (mNGS) library preparation (Supplementary Materials 3), using Illumina Nextera XT paired-end protocol (Illumina, San Diego, CA, USA), as previously described [10,11] and sequenced on iSeq 100 instrument in a single 17.5-h run providing 2 × 150-base pair (bp) long reads. The NGS generated sequences were assembled by Spades on-line software available on Galaxy/Europe bioinformatics (https://usegalaxy.eu, accessed on 17 June 2020) and mapped with CLC Genomics Workbench software version 7.5.0 (Qiagen).

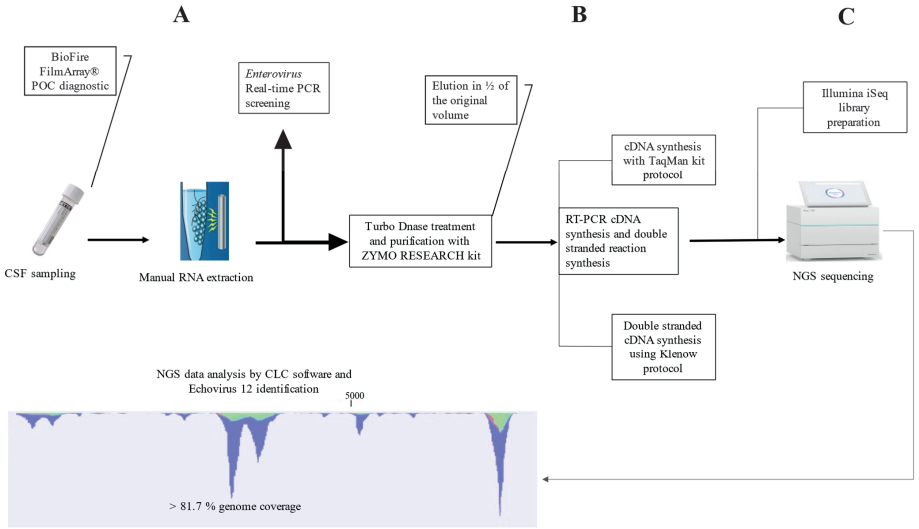

Figure 1. *Echovirus* 12 whole genome sequencing direct diagnosis workflow. The total duration of the handling process is less than 24 h including al steps. (**A**) CSF sampling by lumbar puncture, followed by specific point-of-care (POC) enterovirus

(EVs) diagnosis in FilmArray® CSF direct test (BioFire Diagnostics, Salt Lake City, UT, USA). The viral RNA was extracted and purified following an in-house developed protocol, using QIAamp Viral RNA Mini Kit (Qiagen) and RNA was purified by magnetic DynaBeads (Thermo Fisher, Waltham, MA, USA). The EVs POC diagnostic was confirmed by real-time PCR using LightCycler Multiplex RNA Virus Master kit (Roche Diagnostics®, Mannheim, Germany). (**B**) The extracted RNA was treated by Turbo DNase (Thermo Fisher) and purified with (Zymo Research) kit. RT-cDNA synthesis reaction was performed in 50 µL-volume using kit TaqMan (Thermo Fisher), followed by double strand synthesis using DNA Polymerase I, Large (Klenow) Fragment (BioLabs). (**C**) The double stranded cDNA sequenced in 150-cycle iSeq Illumina instrument following Nextera NGS library preparation iSeq protocol. Finally, NGS data analysis was performed using CLC Genomics Workbench software version 7.5.0 (Qiagen), and more than 81.68% of *Echovirus* 12 genome was obtained directly by next-generation sequencing.

3. Results and Discussion

BLAST analysis of the contigs generated by mNGS after assembling reads with Spades (https://usegalaxy.eu, accessed on 17 June 2020), yielded as best match the *Echovirus 12* strain Travis 2–85 gene (GenBank accession number AF295499.1). This strain was originally isolated from a 6-year-old healthy American male, caused cytopathic effect in tissue culture, was not neutralized by poliomyelitis antiserum, and failed inducing disease in infant mice [12]. As the complete genome of this strain was not available in the GenBank database, the *Echovirus 12* complete genome, prototype Travis (X77708.1) was used as reference sequence for mapping of total reads by CLC Genomics Workbench software. The iSeq sequencing generated 114.818 reads, and 76.284 (66.4%) reads could be mapped on the *Echovirus 12* genome, generating 7 contigs (GenBank accession number; PRJEB39568) covering 6,127 bp, hence 81.7% of this genome (Figure 1). Phylogenetic analysis based on VP1 gene and 3D polymerase encoding genes identified that these sequences belong to an *Echovirus 12* as supported by bootstrap values of 98% and 97%, respectively (Figure 2, Supplementary Materials).

Using this approach of whole genome sequencing, *Echovirus 12* was obtained in one-shot protocol directly from CSF sample. To confirm the NGS result, two *Echovirus 12*-specific primers were designated and used to target a 291-bp *Echovirus 12* genome fragment. The same strain of *Echovirus 12* was identified by sequencing of the amplified fragment at 98.44% sequence identity (not published data).

Echovirus 12 has been detected in patients with diarrhea and aseptic meningitis [9,13]. In this study, we described for the first time a clinical case of *Echovirus 12* meningitis diagnosed by near whole genome sequencing of an *Echovirus 12* directly from CSF by mNGS. This strategy proves high sensitivity in enterovirus detection, which warrants its introduction for routine diagnostic of enterovirus meningitis in addition to viral genomic surveillance and may even be considered for POC laboratories if the very fast Oxford Nanopore Technology is used. Current routine diagnostic targets a short sequence covering around 7% of the *Enterovirus* genome, so that is not indicative for *Enterovirus* genotyping [11]. The availability of the genome instead of a gene fragment necessarily provides improved information regarding typing of the viral strain and identifying mutations and recombinations, and correlating these genotypic features with epidemiological and/or clinical ones. Another important benefit of mNGS upon qPCR or Sanger sequencing is its versatility. Indeed, this is not a targeted approach but instead it is an opened approach that can potentially detect sequences from any virus or microorganism provided these are in sufficient amount; this is thus of particular interest in cases when no infectious agent could be diagnosed. In addition, NGS cost per clinical sample is currently in the same order of magnitude than that of Sanger sequencing. Indeed, we have estimated that current cost per nucleotide is EUR 0.3 for Sanger sequencing and EUR 0.03 for NGS sequencing with Illumina technology; such cost cannot be directly extrapolated to other laboratories due to highly variable cost components, among which the commercial policy of suppliers or the infrastructure of the laboratory. Overall, the present case exemplifies the powerfulness of mNGS in the setting of the routine diagnosis of meningitis.

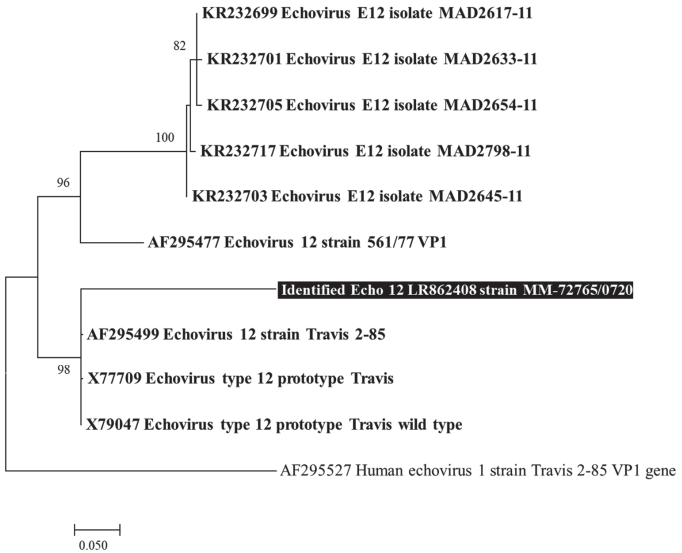

Figure 2. Phylogenetic analysis based on *VP1* gene. The *Echovirus 12* near full-length genome obtained here by mNGS (GenBank accession no LR862408; indicated by a white bold font and a black background, and 9 hit blast *Echovirus 12* recovered from the NBCI GenBank nucleotide sequence database (http://www.ncbi.nlm.nih.gov/nucleotide/, accessed on 29 April 2021), indicated by a bold font, were incorporated in the phylogeny reconstruction in addition to *Echovirus 1* sequence recovered from GenBank database. The sequence obtained in the present study is most similar to *Echovirus 12* Travis stains (X79047, X77709, AF295499) and are clustered with this sequence, confirming the BLAST result. The evolutionary history was inferred in the MEGA 7 software version 7.0.2. This analysis involved 11 nucleotide sequences. There were a total of 548 positions in the final dataset. The tree was performed by applying the neighbor-joining method and the Kimura 2-parameter method. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches. The tree was drawn to scale, with branch lengths in the same units as the evolutionary distances used to infer the phylogenetic tree; the scale bars represent the corresponding number of nucleotide substitutions per site. Bootstrap values $\geq$ 75% are indicated at the nodes.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/pathogens10050610/s1, Supplementary Material 1: Phylogenetic tree based on 3D polymerase encoding gene analysis. Supplementary Material 2: DNase treatment and cDNA synthesis. Supplementary Material 3: NGS Library preparation.

Author Contributions: M.M.: contributed to experimental design, realization of the work, data analysis, interpretation, and writing. C.Z., performed routine analyses and collected clinical information and writing. A.M., clinical diagnostic, and CSF sampling. P.C., Bioinformatic data analysis, interpretation, and writing. M.D., contributed to critically reviewing the manuscript, data interpretation, coordinated and directed the work. All authors have read and agreed to the published version of the manuscript.

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Institutional Review Board Statement: All data were generated as part of routine work at the Assistance Publique-Hôpitaux de Marseille (Marseille University hospitals), and this study is the result of routine clinical management, validated by the IHU Méditerranée Infection Ethics Committee under the following numbers (N: 2021–004). No specific clinical sampling has been done in this study.

Informed Consent Statement: Patient tutors' consent was waived as the study is reporting a diagnostic activity which did not require any specific clinical sample, which result did not modify the medical management of the patient; who cannot be identified through the case report.

Data Availability Statement: The identified *Echovirus 12* genome available on NCBI GenBank accession number; PRJEB39568.

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Conflicts of Interest: The authors have no conflict of interest to declare.

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Chapitre IV : Diagnostic par Métagénomique en Temps Réel (RTM) des méningites au POC.

Préambule

Les méningites bactériennes sont des urgences vitales puisque 50% des cas progressent vers le décès dans les 24-48 heures suivant l'infection en absence de traitement, entraînant une mortalité globale de 8% à 15 % (4,14,15,17). Chaque année, environ 1.3 million de cas de méningites bactériennes sont rapportés dans le monde, estimés à 16 millions de cas entre 1995 et 2013 (15). Les principaux pathogènes dans cette situation sont : *Escherichia coli, Streptococcus agalactiae, Streptococcus pneumoniae, Haemophilus influenzae, Neisseria meningitidis, Listeria monocytogenes* and *Staphylococcus aureus* (71). En 2017 plus de 50% de mortalité des méningites sont dues à *S. pneumoniae*, *N. meningitidis, H. influenzae* et *S. agalactiae*, dont plus de 290.000 cas sont rapportés à l'OMS (14). La culture du LCR est le gold standard pour le diagnostic de méningites bactériennes (24), malgré entre 10% et 60% d'échecs (15). En parallèle, le diagnostic d'urgence au laboratoire POC est basé sur l'amplification multiplexée de séquences spécifiques (28), éventuellement complété par l'amplification-séquençage du gène 16S rRNA (1). Ensuite, le profil de sensibilité et résistance aux antibiotiques ne peut être déterminé *in vitro* que pour les bactéries isolées par culture (15,28). Il en va de même pour le sérotypage des souches de *Neisseria meningitidis* et *Haemophilus influenzae* (17), dont le résultat peut guider la vaccination en prophylaxie secondaire (37).

Dans le travail expérimental exposé dans ce chapitre, nous avons mis au point un protocole de séquençage direct de l'ADN bactérien dans le LCR et son analyse avec comme "cahier des charges", de ne pas dépasser une demi-journée de la réception de prélèvement LCR jusqu'à la remise de résultat. Après l'extraction automatique d'ADN par EZ1 (Qiagen, Courtaboeuf, France), une librairie suivant le protocole Oxford Nanopore Technologies (Oxford, Grande Bretagne) a été préparée puis séquencée dans l'instrument Oxford Nanopore MinION. Les données générées par MinION sont analysées en temps réel par blast afin d'identifier l'agent pathogène causatif. Un point-clé est la constitution d'une database syndromique locale adaptée à l'épidémiologie locale, mais enrichie et adaptée au fur et à mesure des résultats nouveaux, en parallèle de la database généraliste Oxford Nanopore. Ce travail à fait objet de quatre articles : nous avons commencé par la mise au point, puis nous avons analysé prospectivement une série de 52 LCR pour valider cette approche nouvelle du diagnostic. L'implantation de cette approche au POC peut permettre une surveillance génomique en temps réel des variants des agents pathogènes responsables de méningite circulant dans la zone d'étude, afin de définir une nouvelle stratégie de contrôle épidémiologique et de vaccination. D'autres développements pourraient inclure l'application de la métagénomique en temps réel sur des cas non documentés et des cas de virus ARN afin d'enrichir le répertoire des agents pathogènes responsables de méningite communautaires, non couramment diagnostiqués en cas d'infection de système nerveux central.

Article 5

Haemophilus influenzae Meningitis Direct Diagnosis by Metagenomic Next-Generation Sequencing: A Case Report.

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Brief Report

Haemophilus influenzae Meningitis Direct Diagnosis by Metagenomic Next-Generation Sequencing: A Case Report

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Abstract: Current routine real-time PCR methods used for the point-of-care diagnosis of infectious meningitis do not allow for one-shot genotyping of the pathogen, as in the case of deadly *Haemophilus influenzae* meningitis. Real-time PCR diagnosed *H. influenzae* meningitis in a 22-year-old male patient, during his hospitalisation following a more than six-metre fall. Using an Oxford Nanopore Technologies real-time sequencing run in parallel to real-time PCR, we detected the *H. influenzae* genome directly from the cerebrospinal fluid sample in six hours. Furthermore, BLAST analysis of the sequence encoding for a partial DUF417 domain-containing protein diagnosed a non-b serotype, non-typeable *H. influenzae* belonging to lineage *H. influenzae* 22.1-21. The Oxford Nanopore metagenomic next-generation sequencing approach could be considered for the point-of-care diagnosis of infectious meningitis, by direct identification of pathogenic genomes and their genotypes/serotypes.

Keywords: bacterial meningitis; point-of-care diagnostic; *Haemophilus influenzae*; Oxford Nanopore Technologies; real-time sequencing; metagenomic next-generation sequencing

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1. Introduction

The microbiological diagnosis of bacterial meningitis presently carried out in point-of-care (POC) laboratories is based on different methods of detection of nucleotide sequences specific to the target pathogen [1,2]. More specifically, this detection is based on techniques using polymerase chain reaction (PCR) amplification by target of pathogen-specific genomic sequences, with the product of the amplification being detected by fluorescence in the so-called real-time PCR (RT-PCR) modality [2–4], permitting the detection of the presence or absence of pathogenic genomes, which is not sufficient to carry out pathogen genotyping.

Here, we report one case of *Haemophilus influenzae* meningitis that was diagnosed by metagenomics next-generation sequencing (mNGS), using the technology developed by Oxford Nanopore Technologies (Oxford Nanopore, Oxford Science Park, UK) directly from a cerebrospinal fluid (CSF) sample, identifying the pathogen genome, based on real-time sequencing by comparison to the Oxford Nanopore online database using EP2ME online software.

2. Case Report and Methods

A 22-year-old male patient presented to the emergency room of the neurosurgery department at the North Hospital of Marseille following a more than six-metre fall. The patient presented with numerous contusions and a dislocation orbito-naso-ethmoido-frontal type panfacial trauma complicated by subdural haematoma and subarachnoid haemorrhage. At his arrival in the emergency room, the patient presented a Glasgow score of 5, blood pressure of 140/70, cardiac frequency of 110 bpm and 75% saturation in ambient

air. Ten days after his admission, the patient presented with sudden onset of febrile (39 °C) meningeal syndrome. Cerebrospinal fluid (CSF) analysis showed a leukocyte count of 198 cell/mm^3 (37% neutrophils, 63% lymphocytes), erythrocyte count of 4200 cell/mm^3, protein at 3.31 g/L and glucose at 0.33 mmol/L. Direct microscopic examination after Gram staining did not reveal the presence of any bacteria. A BioFire FilmArray® assay (Biofire bioMérieux, Marcy-l'Etoile, France), performed as previously described [1], yielded *Haemophilus influenzae* according to the meningitis encephalitis panel. The patient was treated with antibiotics for 14 days, 2 days with meropenem (4 g/day) and linezolid (1.2 g/day), 2 days with meropenem only, 2 days with cefotaxime and finally 8 days with amoxicillin at 12 g/day. A drastic decrease in the leukocyte count was observed from the second day of antibiotherapy treatment. The patient suffered cognitive, memory and consciousness disorders and left eye blindness.

In parallel to the BioFire FilmArray® diagnostic, total DNA was extracted from a 200 µL CSF sample after a 20-minute incubation with proteinase K at 37 °C using an EZ1 DNA Kit (Qiagen, Courtaboeuf, France) and eluted in a 50 µL final volume. For direct microbial genome sequencing, 1 µg of DNA was incorporated into an Oxford Nanopore MinION library preparation according to the manufacturer's protocol (https://community.nanoporetech.com/protocols/). The library was quantified and normalized in a 47 µL volume using a QubitTM fluorometer using a Qubit dsDNA High Sensitivity Assay Kit (Life Technology, Villebon-sur-Yvette, France). Briefly, DNA repair and end preparation were performed in a 60 µL final volume containing 47 µL of prepared DNA, 1 µL of DNA CS (DNA control), 3.5 µL of NEBNext FFPE DNA Repair buffer, 2 µL of NEBNext FFPE DNA Repair mix (New England BioLabs, Evry-Courcouronnes, France), 3.5 µL of Ultra II End-prep reaction buffer and 3 µL of Ultra II End-prep enzyme mix (New England BioLabs). The repair reaction was performed in a GeneAmp PCR System Thermal Cycler (Applied Biosystems, Foster City, CA, USA) for 5 minutes at 20 °C followed by a 5 min incubation at 65 °C. Repaired DNA was purified using an equal volume of Agencourt Ampure XP beads (Beckman Coulter, Villepinte, France) in the presence of 70% ethanol and then eluted in 61 µL of distilled water. The adapter ligation step was performed in a 100 µL volume containing 60 µL of purified DNA, 25 µL of ligation buffer, 10 µL of T4 DNA ligase (New England BioLabs) and 5 µL of the adapter mix and incubated for 10 minutes at room temperature. A second wash was carried out using Agencourt Ampure XP beads (Beckman Coulter) in a ratio of 0.4. The final library was recovered in a 15 µL final volume, diluted in 75 µL of flow cell loading mix and sequenced for 4 hours on a MinION instrument (Oxford Nanopore). Real-time analysis of sequencing data was performed using EPI2ME software (version 2019.11.11-2920621). A second analysis was performed using Kraken 2 (https://ccb.jhu.edu/software/kraken2/) and visualized by Pavian (https://fbreitwieser.shinyapps.io/pavian/).

3. Results and Discussion

After running for 4 h, MinION sequencing generated 202,010 reads including 1277 unclassified reads. Real-time EPI2ME data analysis yielded 192,260 human genome reads, 6598 *Escherichia coli* (control) reads, 29 *Shigella* reads, 23 *Lambdavirus* reads and 11 (0.00005%) reads corresponding to the *H. influenzae* genome. The *Shigella* and *Lambdavirus* reads come from repair and ligation enzymes used in the library preparation. Kraken online analysis and visualization by Pavian online software showed only *H. influenzae* with high stringency (Figure 1). The BLAST analysis of the longest 1298 bp sequence encoding for partial the aerobic respiration control sensor protein *ArcB* gene after specific *H. influenzae* read extraction using Kraken Tools yielded a non-b serotype, non-typeable *H. influenzae* strain P641-4342 with 97% sequence identity (GenBank accession number CP031687.1) belonging to *H. influenzae* lineage 22.1-21 (http://www.iedb.org/sourceOrgId/374927) [5]. This finding has been routinely validated by positive specific *H. influenzae* real-time PCR at 30 Ct using a LightCycler® 480 thermal-cycler (Roche, Wilmington, NC, USA) in a 20 µL final reaction volume containing 5 µL DNA and Takyon No Roxe Probe MasterMix (Eurogentec),

targeting a 167 bp fragment length of the *ompP1* gene as previously described [6]. While the patient had been vaccinated for *H. influenzae* serotype B, whole genome sequencing identified a non-typeable *H. influenzae* serotype (NTHi), against which the patient was not immunized. The in silico antibiotic susceptibility pattern derived from the whole genome by ResFinder online software (Version 4.1) predicted *H. influenzae* strain P641-4342 to be susceptible to all antibiotics, as experimentally confirmed by an in vitro antibiogram (File S1).

This new approach has been proposed for real-time pathogenic genome detection, essentially based on the time of manipulation of approximately 6 hours in total and its sensitivity to detect microbial genomes, even at low levels. As a novel approach for POC diagnosis, direct identification of the pathogenic genome by metagenomic next-generation sequencing remains a challenge for routine diagnosis. Utilization of Oxford Nanopore technology in mNGS allows real-time sequencing analysis to be performed directly from clinical samples [7]. The *H. influenzae* genome was the first whole bacterial genome to be sequenced [8]. In this case, we diagnosed *H. influenzae* directly from a CSF sample for the first time in our laboratory using Oxford Nanopore Technologies sequencing. Using metagenomics real-time sequencing allowed us to identify an *H. influenzae* non-b serotype lineage 22.1-21 sensitive to all antibiotics by online analysis using ResFinder software in less than 6 hours, which was confirmed by solid culture after three days in routine bacteriology laboratory diagnostics. As a new alternative for POC diagnostics, this strategy will be suitable for implementation in routine diagnostics of CNS infection and genomic surveillance in infectious diseases, despite the variability of genome coverage depending on the pathogenic charge in the CSF.

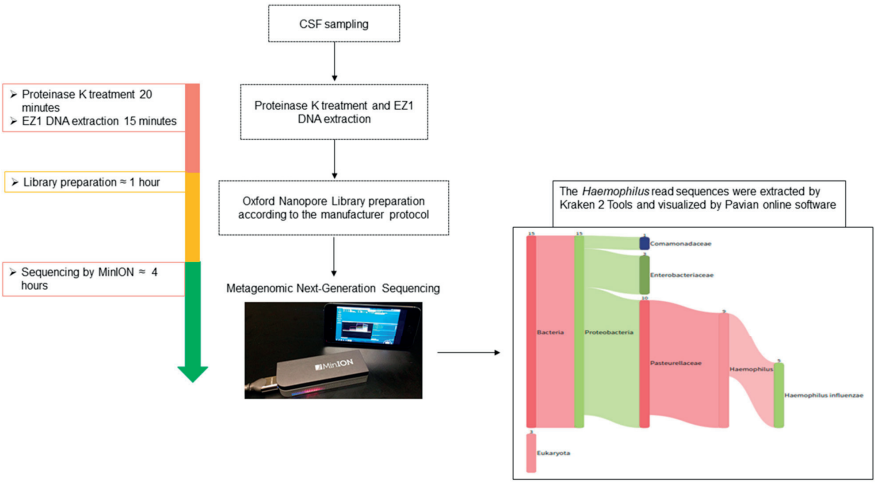

Figure 1. POC real-time sequencing diagnosis of *H. influenzae* meningitis by using Oxford Nanopore Technology. The total process duration was less than 6 h. The cerebrospinal fluid was treated with proteinase K for 20 min at 56 °C, and total DNA was eluted in a 50 µL volume. The Oxford Nanopore library was prepared according to the manufacturer's protocol and diluted in a 75 µL final volume of flow cell loading mix. The diluted library was sequenced on a MinION instrument, and MinION sequencing data were analysed in real time using EPI2ME software. They were extracted by Kraken 2 Tools and visualized by Pavian online software.

4. Conclusions

Based on the simplicity, rapidity and sensitivity of mNGS real-time sequencing, we are now implementing Oxford Nanopore sequencing technology in the POC laboratory for the rapid diagnosis of bacterial meningitis, providing additional pieces of information over routinely used syndromic real-time PCR kits with which mNGS is competing in terms of delay and cost.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/pathogens10040461/s1, File S1: Antibiogram analysis.

Author Contributions: M.M., contributed to experimental design, realisation of the work, data analysis, interpretation and writing. Q.K., collected samples, performed routine analyses, collected clinical information and writing. J.D., bioinformatics data analysis. P.-H.R. and L.T., clinical diagnostic and CSF sampling. M.D., contributed to critically reviewing the manuscript, data interpretation and coordinated and directed the work. All authors have read and agreed to the published version of the manuscript.

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Institutional Review Board Statement: All data were generated as part of routine work at the Assistance Publique-Hôpitaux de Marseille (Marseille University hospitals), and this study is the result of routine clinical management. No specific clinical sampling has been done in this study.

Informed Consent Statement: Written informed consent was obtained from the patient for publication of this case report and any accompanying images. A copy of the written consent is available for review by the Editor-in-Chief of this journal.

Data Availability Statement: All the identified genomics sequences have been deposited and are available on the official website of IHU Méditerranée Infection via the following links. https://www.mediterranee-infection.com/acces-ressources/donnees-pour-articles/direct-diagnosis-by-whole-genome-sequencing-of-haemophilus-influenzae-meningitis/. GenBank accession bioproject: PRJNA702049.

Conflicts of Interest: The authors have no conflict of interest to declare.

List of Abbreviations

dsDNA	double-strand desoxyribonucleic acid.
RT-PCR	real-time polymerase chain reaction.
POC	point-of-care.
CSF	cerebrospinal fluid.
mNGS	metagenomic next-generation sequencing.

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Article 6

Real-time whole genome sequencing direct diagnosis of *Streptococcus pneumoniae* meningitis: a case report.

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Fournier P.E. [1, 2, 3], Drancourt M[1, 2, 3].

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Letter to the Editor

Real-time whole genome sequencing direct diagnosis of *Streptococcus pneumoniae* meningitis: A case report

Dear editor,

As reported in this Journal recently, bacterial meningitis is a life-threatening invasive infection that could progress to mortality within 24 h in the absence of an appropriate treatment, causing annually more than 50% deaths from all-cause meningitis and leaving one in five people recovering with chronic neurological disorders.[1] Current rapid point-of-care (POC) diagnosis of community acquired bacterial meningitis is based on PCR-based multiplex assays detecting pathogen-specific sequences in the cerebrospinal fluid (CSF). [2] These assays however do not provide all the pieces of information desirable for the optimal medical management of the patient clinically diagnosed with community-acquired meningitis. Antibiotic susceptibility to finely tune the antibiotic treatment and genotyping to guide source tracing. Real-time whole genome sequencing is an alternative approach recently developed for detecting and genotyping pathogens directly from clinical samples, and to determine antibiotic susceptibility profile, in real time. [3]

Here, we reported a 69-year-old woman was admitted to the emergency department with a 38.5 °C fever which had lasted a day, repeated dizzy spells, and vomiting. Clinical examination disclosed neck stiffness, headache, and photophobia, without rash or purpura. Biological testing found an inflammatory syndrome with C-reactive protein at 350 mg/L. CSF analyses yielded hyperproteinorrhachia at 1.16 g/L and hypoglycorrhachia at 0.11 mmol/L, a leukocyte count of 4 cells/mm^3 and erythrocytes at 50 cell/mm^3. Gram staining showed Gram-positive diplococci identified as *S. pneumoniae* using a real-time multiplex PCR assay (bioFire FilmArray® Meningitis/Encephalitis panel, bioMérieux, Marcy-l'Etoile, France) at the POC laboratory.[2] The cultured *S. pneumoniae* was categorized *in vitro* susceptible to penicillin G, ceftriaxone, erythromycin, doxycycline, and chloramphenicol by Mueller-Hinton culture antibiogram after two days. The patient was treated with ceftriaxone, 6 g/day for 14 days and dexamethasone, 40 mg/day for five days. Further evaluations found hyperproteinemia at 110 g/L and a plasmatic monoclonal peak of IgG kappa quantified at 39 g/L (Capillarys, Sebia, Evry, France), which led to a subsequent diagnosis of multiple myeloma.

Alongside the bioFire FilmArray® investigation, total DNA was extracted from 200 µL of cerebrospinal fluid using the EZ1 DNA Kit and an EZ1 automaton (Qiagen, Courtaboeuf, France) following 20 min incubation with proteinase K at 56 °C and eluted in a 50 µL final volume. Next-generation sequencing was performed using a MinION device (Oxford Nanopore Technology, Oxford, UK), as previously described,[3] using 47 µL of the extracted DNA for the MinION library preparation (Appendix 1). The final library was recovered in 15 µL volume and was further diluted in 75 µL of flow cell loading mix and sequenced for 20 min on a MinION instrument. Sequencing data were analyzed in real-time using the EPI2ME online software (version 2019.11.11-2920621). In a second analysis, we assembled and mapped the generated reads using CLC Genomics Workbench software version 7.5.0 (Qiagen). Furthermore, 1 ng of DNA was used for NGS sequencing using the Illumina Nextera XT library preparation and the paired-end protocol (Illumina, San Diego, USA), as previously described,[4] on an iSeq 100 instrument (Illumina) and sequences were analyzed using the CLC Genomics Workbench software (Qiagen).[5]

A twenty-minute MinION sequencing run generated 61,150 reads, including 11,659 *S. pneumoniae* reads (Fig. 1). Assembly process generated 92.5% genome coverage and further blast analysis yielded *S. pneumoniae* with 98.97% sequence identity with the reference *S. pneumoniae* strain A6-10, a serotype 10-A (GenBank accession no. CP053210.1). The clinical strain was *in silico* susceptible to all routinely used antibiotics as determined using the ResFinder bio-tool (http://cge.cbs.dtu.dk/services/ResFinder/) (Appendix 2) (Fig. 1). MinION sequencing data were confirmed by Illumina iSeq sequencing, generating 503,192 reads. Blast comparison of the 73,597-bp longest contig identified a best match with *S. pneumoniae* strain A6-10 with 98.97% sequence similarity. A total of 338,879/503,192 (67.35%) reads mapped to the reference sequence, identifying 90% of the *S. pneumoniae* genome. Further online analysis using Multilocus Sequence Typing (MLST), revealed a perfect match with the *S. pneumoniae* reference genome, sharing common characteristics with the 128 isolates disponible in the GenBank database, including 104 isolates from European countries of which 58 were serotype 10A, all of them being susceptible to all antibiotics as confirmed by routine bacterial culture (Appendix 2).

S. pneumoniae meningitis is a life-threatening invasive infection warranting its rapid complete diagnosis.[6,7] Diagnosis methods currently available at the POC laboratory, do not provide pieces of information regarding typing and antibiotic susceptibility of the detected *S. pneumoniae*.[2] Here, we successfully applied direct near whole genome real-time sequencing diagnosis of community-acquired *Streptococcus pneumoniae* meningitis using MinION technology, providing in less than two-hour workflow including only 20 min sequencing time, the detection, identification, typing and *in-silico* antibiogram; after we developed a specific sequencing procedure as here reported (Fig. 1). The fact that this procedure is time-competitive with that of the real-time PCR multiplex assay, providing valuable additional pieces of information, makes real-time whole genome sequencing a suitable approach for the diagnosis of community-acquired meningitis at the POC laboratory.

Ethical statement

All data were generated as part of routine work at the Assistance Publique-Hôpitaux de Marseille (Marseille University hospi-

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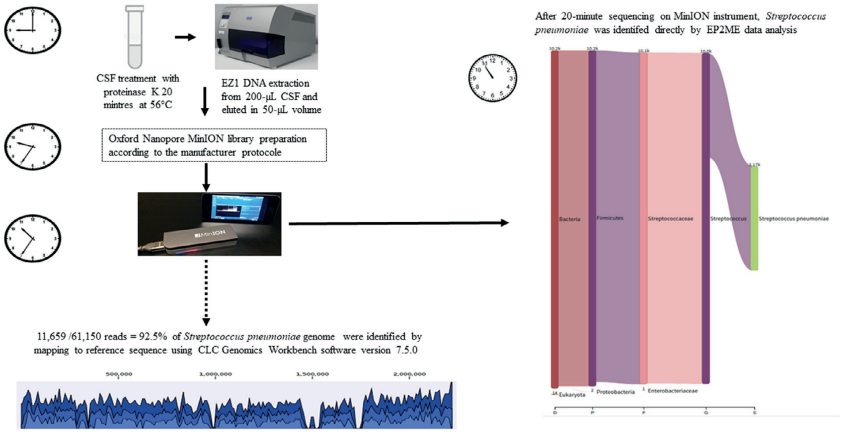

Fig. 1. . Real-time whole genome sequencing of *Streptococcus pneumoniae* (*S. pneumoniae*). Total handling time was less than two hours, including 35 min for cerebrospinal fluid (CSF) processing and EZ1 DNA extraction, 60 min for the Oxford Nanopore library preparation and 20 min for MinION library sequencing. The MinION sequencing result was directly analyzed by EP2ME software, then the generated reads were mapped to the reference genome by CLC software. In this case, the *S. pneumoniae* genome was detected after a 20 min sequencing run, and the full data analysis yielded 92.5% genome coverage directly recovered from CSF sample.

tals), and this study is the result of routine clinical management. This study was approved by our Institute's Ethics Committee under number (2021-004). No specific clinical sampling was performed in this study.

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Madjid Morsli is a PhD student supported by the Fondation Méditerranée Infection. This work was supported by the French Government under the Investissements d'Avenir (Investments in the Future) program managed by the Agence Nationale de la Recherche (ANR,fr: National Agency for Research) [reference: Méditerranée Infection 10-IAHU-03].

Declaration of Competing Interest

The authors have no conflicts of interest to declare.

CRediT authorship contribution statement

M. Morsli: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Software, Writing – original draft. **Q. Kerharo:** Formal analysis, Writing – original draft. **S. Amrane:** Writing – original draft. **P. Parola:** Investigation, Writing – original draft. **P.E. Fournier:** Conceptualization, Project administration, Writing – original draft. **M. Drancourt:** Conceptualization, Supervision, Validation, Visualization, Writing – original draft, Writing – review & editing.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.jinf.2021.10.002.

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Article 7

Direct diagnosis of *Pasteurella multocida* meningitis using next-generation sequencing.

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Direct diagnosis of *Pasteurella multocida* meningitis using next-generation sequencing

We recently implemented a real-time metagenomic sequencing approach (Oxford Nanopore Technologies, Oxford, UK) for a community-acquired, life-threatening case of meningitis diagnosed in a point-of-care laboratory,[1] to complement point-of-care diagnosis by PCR-based multiplex assays.[2] Here we describe how real-time metagenomic sequencing enabled the direct diagnosis of meningitis caused by *Pasteurella multocida*, which is not routinely detected by current point-of-care assays, from cerebrospinal fluid (CSF) collected from a patient.

A 77-year-old woman was admitted to the emergency department for brutal onset of febrile delirium (39°C). At that time, blood tests showed a high concentration of C-reactive protein (213 mg/L), and the patient was treated with ceftriaxone (1 g/day) until blood cultures collected at admission grew *P multocida*. The patient reported a domestic cat bite to the left ankle 5 days before admission, which was treated with pristinamycin immediately after the event and then with cefotaxime (4 g, four times a day) and intravenous ciprofloxacin (400 mg, three times a day) in a first hospital. Analysis of the CSF showed purulent meningitis, with 5·48 g/L proteins, 0·54 g/L glucose, 16 g/L lactates, 6300 white blood cells per µL, and 800 red blood cells per µL. All routine microbiological assays—including direct examination of the CSF by means of Gram staining, culture, the BioFire FilmArray assay (bioMérieux, Marcy-l'Etoile, France), and 16S rRNA amplification—remained negative. Total DNA was extracted from 150 µL CSF by means of the EZ1 DNA Tissue Kit (Qiagen, Courtaboeuf, France) and eluted in a volume of 50 µL. Then, 47 µL DNA was incorporated into the Oxford Nanopore library as previously described,[1] sequenced on an Oxford Nanopore MinION (Oxford Nanopore Technologies, Oxford, UK) sequencer for 5 h, and sequences were analysed in real-time using the EPI2ME online software (Oxford Nanopore Technologies, Oxford, UK). Seven *P multocida*-specific reads were generated over 5000 reads in a 30 min run, and 53 *P multocida* reads were generated over 32 000 reads after 2 h. Read assembly and Basic Local Alignment Search Tool analysis using the CLC Genomics Workbench software version 21.0.3 (Qiagen, Redwood City, CA, USA) mapped a total of 288 of 159 420 reads to the *P multocida* reference sequence (NZ_CP028927). In total, 168 260 nucleotides (7·3%) of the *P multocida* genome were obtained directly from the CSF. In silico analysis of antibiotic-resistance genes using the ResFinder biotool showed that this strain of *P multocida* was susceptible to all tested antibiotics.

Pasteurellosis, a zoonotic infection caused by *Pasteurella* bacteria, can arise as a complication of domestic animal bites. The infection causes bacteraemia, which in rare cases can progress to purulent meningitis if the pathogen penetrates the blood–brain barrier.[3,4] *P multocida* is not routinely detected by current point-of-care assays used to diagnose community-acquired meningitis, such as the one reported by Vincent and colleagues.[2] Real-time metagenomic sequencing of total DNA extracted from the CSF was found to be a suitable alternative method for detecting the pathogen genome directly without a specific gene target, and provided additional information on the genotype.[1] This technology opens new perspectives for the microbiological diagnosis of life-threatening and non-routinely diagnosed infections, as well as for the genomic surveillance of pathogens that are responsible for infectious diseases.

All data were generated as part of routine work at the Assistance Publique-Hôpitaux de Marseille (Marseille University Hospitals), and this study is the result of routine clinical management. This study was approved by the Ethics Committee of the IHU Méditerranée Infection, Marseille, France under number 2021-004. No specific clinical sampling was done in this study. MM is a PhD student supported by the Fondation Méditérranée Infection. This work (laboratory material and reactives) was supported by the French Government under the Investissements d'Avenir (Investments in the Future) programme managed by the Agence Nationale de la Recherche (ANR; National Agency for Research; reference: Méditerranée Infection 10-IAHU-03). MM contributed to the experimental design, carried out the work, analysed the data, interpreted the data, and wrote the paper. YB collected samples. OC and AT did routine analyses, clinical diagnoses, CSF sampling, and collected clinical information. PEF, LH, and MD contributed to the critical review of the manuscript and the interpretation of the data, and coordinated and directed the work. All authors declare that they have read and approved the manuscript. We declare no competing interests.

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For **ResFinder biotool** see http://cge.cbs.dtu.dk/services/ResFinder/

Article 8

Real-time metagenomics-based diagnosis of community-acquired meningitis: a prospective series, Southern France.

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Abstract:	Background. Point-oc-care (POC) diagnosis of life-threatening community-acquired meningitis currently relies on multiplexed RT-PCR assays, that lack genotyping and antibiotic susceptibility profiling. We assessed the usefulness of real-time metagenomics (RTM) directly applied to the cerebrospinal fluid (CSF) for the identification, typing and susceptibility profiling of pathogens responsible for community-acquired meningitis. Methods A series of 52 CSF samples from patients suspected of having community-acquired meningitis, were investigated at POC by direct RTM in parallel to routine real-time multiplex PCR (RT-PCR) and bacterial culture, for the detection of pathogens. RTM-generated sequences were blasted in real-time against an in-house database incorporating the panel of 12 most prevalent pathogens and against NCBI using EPI2ME online software, for pathogen identification. In-silico antibiogram and genotype prediction were determined using the ResFinder bio-tool and MLST online software. Finding Over eight months, routine multiplex RT-PCR yielded 49/52 positive CSFs, including 21 Streptococcus pneumoniae, nine Neisseria meningitidis, eight Haemophilus influenzae, three Streptococcus agalactiae, three Herpesvirus-1, two Listeria monocytogenes, and one each of Escherichia coli, Staphylococcus aureus and Varicella-Zoster Virus. Parallel RTM agreed with the results of 47/52 CSFs and revealed two discordant multiplex RT-PCR false positives, one H. influenzae and one S. pneumoniae. Both multiplex RT-PCR and RTM agreed on the negativity of three CSFs. While multiplex RT-PCR routinely took 90 minutes, RTM took 120 minutes, although the pipeline analysis detected the pathogen genome after 20 minutes of sequencing in 33 CSF samples; and after two hours in 14 additional CSFs; yielding > 50% genome coverage in 19 CSFs. RTM identified 14 pathogen genotypes, including a majority of H. influenzae b, N. meningitidis B and S. pneumoniae 11A and 3A. In all 16 cultured bacterial pathogens, the in-silico antibiogram agreed with the in-vitro antibiogram, available within 48 hours. Interpretation. In addition to pathogen detection, RTM applied to CSF samples offered supplementary information on bacterial profiling and genotyping. These data provide

	the proof-of-concept that RTM could be implemented in a POC laboratory for one-shot diagnostic and genomic surveillance of pathogens responsible for life-threatening meningitis.

Research in context

Evidence preceding this study

Community-acquired bacterial meningitis is a life-threatening infection that can progress to mortality within 48 hours. The emergency diagnosis of infectious meningitis is currently based on multiplex real-time amplification using a syndromic panel limited by the most frequent microorganisms which lead to a central nervous system prognosis. Bacterial characterisation and drug resistance profiling require additional *in-vitro* investigations which take over 48 hours, delaying pathogen-targeted treatment. Genomic surveillance and antibiotic resistance testing are based only on bacteria isolated from cerebrospinal fluid, failing in 60% of cases. We searched PubMed up to 30 November 2021 for research articles published in English, using the following search terms “real-time metagenomics sequencing”, “meningitis”, and “direct diagnosis”. Several articles had been published testing the Oxford Nanopore technologies sequencing on CSF, but no investigations were found into the direct diagnosis of CSF series. When the three terms were used together, only two articles previously published by us were found. As previously reported, we implemented real-time metagenomic sequencing (RTM) at the POC laboratory for the diagnosis of life-threatening infectious meningitis, in addition to the BioFire FilmArray® investigation. In light of its simplicity, rapidity and additional information collected, we propose RTM as a powerful diagnostic tool for the investigation of prospective series of CSF samples collected from patients with meningitis.

Added value of this study

To the best of our knowledge, here we diagnosed a series of community-acquired meningitis cases by RTM directly from CSF samples. Over eight months, 52 CSFs were investigated

directly by RTM using a four-hour workflow. Thirty-three CSF samples (70.2%) were diagnosed as positive after a 20-minute sequencing run and an additional 14 were diagnosed as positive after two hours. The pipeline analysis of antibiotic resistance and bacteria genotyping was provided *in-silico* at the same time as sequencing, in contrast to conventional diagnostics. In addition, uncultured bacteria were successfully profiled *in-silico*, basing on pathogen genome analysis, independently of genome coverage.

Implications of the available evidence

Despite the limited sample size in this study, using a four-hours workflow, RTM proved successful in diagnosing, genotyping, and profiling bacteria directly from CSF samples. At one discordance with conventional multiplex RT-PCR, RTM is a suitable method for the diagnosis of life-threatening meningitis in a POC laboratory.

Real-time metagenomics-based diagnosis of community-acquired meningitis: a prospective series in southern France.

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Abstract

Background Point-of-care (POC) diagnosis of life-threatening community-acquired meningitis currently relies on multiplexed RT-PCR assays which lack genotyping and antibiotic susceptibility profiling. We assessed the usefulness of real-time metagenomics (RTM) directly applied to the cerebrospinal fluid (CSF) for the identification, typing and susceptibility profiling of pathogens responsible for community-acquired meningitis.

Methods A series of 52 CSF samples from patients suspected of having community-acquired meningitis, were investigated at the POC using direct RTM alongside routine real-time multiplex PCR (RT-PCR) and bacterial culture, for the detection of pathogens. RTM-generated sequences were blasted in real-time against an in-house database incorporating a panel of the 12 most prevalent pathogens and against NCBI using EPI2ME online software, for pathogen identification. *In-silico* antibiogram and genotype prediction were determined using the ResFinder bio-tool and MLST online software.

Finding Over eight months, routine multiplex RT-PCR yielded 49/52 positive CSFs, including 21 *Streptococcus pneumoniae*, nine *Neisseria meningitidis*, eight *Haemophilus influenzae*, three *Streptococcus agalactiae*, three *Herpesvirus*-1*,* two *Listeria monocytogenes*, and one each of *Escherichia coli*, *Staphylococcus aureus* and Varicella-Zoster Virus. The parallel RTM agreed with the results of 47/52 CSFs and revealed two discordant multiplex RT-PCR false positives, one *H. influenzae* and one *S. pneumoniae*. Both multiplex RT-PCR and RTM agreed on the negativity of three CSFs. While multiplex RT-PCR routinely took 90 minutes, RTM took 120 minutes, although the pipeline analysis detected the pathogen genome after 20 minutes of sequencing in 33 CSF samples, and after two hours in 14 additional CSFs; yielding > 50%

genome coverage in 19 CSFs. RTM identified 14 pathogen genotypes, including a majority of *H. influenzae* b, *N. meningitidis* B and *S. pneumoniae* 11A and 3A. In all 16 cultured bacterial pathogens, the *in-silico* antibiogram agreed with the *in-vitro* antibiogram in 12 cases, and the results were available within 48 hours.

Interpretation. In addition to pathogen detection, RTM applied to CSF samples offered supplementary information on bacterial profiling and genotyping. These data provide the proof-of-concept that RTM could be implemented in a POC laboratory for one-shot diagnosis and genomic surveillance of pathogens responsible for life-threatening meningitis.

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Key words: community-acquired meningitis, real-time metagenomics, point-of-care (POC) laboratory diagnosis, antibiotic resistance, genotyping.

Research in context

Evidence preceding this study

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Added value of this study

We diagnosed a series of community-acquired meningitis cases by RTM directly from CSF samples. Over eight months, 52 CSFs were investigated directly by RTM using a four-hour

workflow. Thirty-three CSF samples (70.2%) were diagnosed as positive after a 20-minute sequencing run and an additional 14 were diagnosed as positive after two hours. The pipeline analysis of antibiotic resistance and bacteria genotyping was provided *in-silico* at the same time as sequencing, in contrast to conventional diagnostics. In addition, uncultured bacteria were successfully profiled *in-silico*, basing on pathogen genome analysis, independently of genome coverage.

Implications of the available evidence

Despite the limited sample size in this study, using a four-hour workflow, RTM proved successful in diagnosing, genotyping, and profiling bacteria directly from CSF samples. With only one discordance with conventional multiplex RT-PCR, RTM is a suitable method for the diagnosis of life-threatening meningitis in a POC laboratory.

INTRODUCTION

The rapid diagnosis of life-threatening, community-acquired meningitis (CAM) remains challenging in point-of-care (POC) laboratories.[1] Bacterial meningitis has a 24-hour mortality of 8%–15%,[2] and results in an estimated 290,000 deaths every year, causing more than 50% of all meningitis deaths annually and leaving one in five people recovering with chronic handicap.[3] Community-acquired bacterial meningitis around the world is mainly due to *Streptococcus pneumoniae* (*S. pneumoniae*), *Neisseria meningitidis* (*N. meningitidis*), *Haemophilus influenzae* (*H. influenzae*) and *Streptococcus agalactiae* (*S. agalactiae*).[4] In Europe, *S. pneumoniae* and *N. meningitidis* are the most common causes of bacterial meningitis[2] (22.5% of cases) and children ≤ 5 years represent 47% of cases.[2] Current POC diagnosis of bacterial meningitis is based on real-time multiplex PCR (RT-PCR) assays incorporating a syndromic meningitis and encephalitis panel,[5–10] targeting small, specific pieces of the pathogen genome.[5,6] These approaches overlook serotype/genotype diversity, a major limitation for the microbiological diagnosis of bacterial meningitis and do not provide sufficient information for pathogen genotyping. Furthermore, they require bacterial culture to characterise different serotypes and antimicrobial resistance.[5,6] Accordingly, additional specific PCRs have to be performed for genotyping *N. meningitidis* B and C serotypes,[11–13] and the *H. influenzae* b serotype associated with invasive diseases.[2,14]

Real-time metagenomics sequencing (RTM) could, theoretically, overcome this limitation, identifying the causative agent of meningitis,[15] as well as its genotype/serotype directly from the cerebrospinal fluid (CSF) based on pathogen genome sequence.[14,16] Indeed, we and others have already published evidence that RTM could be implemented in a POC laboratory, for one-shot

diagnosis and genotyping as well as *in-silico* antibiotic resistance prediction, which is competitive in terms of time and cost with commercial multiplex RT-PCR.[14–18]

In this study, we prospectively diagnosed a series of cases of community-acquired meningitis, directly using RTM on left-over CSF samples in a POC laboratory.

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METHODS

Ethics: All data were generated as part of routine laboratory work at the Assistance Publique-Hôpitaux de Marseille and Nîmes university hospital, in the context of the routine clinical management of patients suspected of having community-acquired meningitis. No specific clinical sampling was performed for this study and RTM was applied to anonymised left-over CSF samples. Information on the age and sex of patients were anonymously collected, following a standard routine laboratory protocol including multiplex real-time PCR, which was carried out in full respect of the French law regarding clinical research. Accordingly, this study was approved by the IHU Méditerranée Infection Ethics Committees (under reference number 2021-004) before the study began in Marseille, and further approval was granted by the "Interface Recherche Bioéthique Institutional Review Board" Ethics Committee of Nimes CHU (under reference number 21.0016) before the study began in Nîmes.

Routine microbial diagnosis: In the POC laboratory, all CSF samples were routinely examined to count white and red blood cells directly using the NucleoView NC-3000 equipment and NucleoView/ChemoMetec software (ChemoMetec NucleoCounter, Allerod, Denmark). In parallel, 200 µL samples of CSF were used for multiplex RT-PCR diagnosis (BioFire FilmArray®, bioMérieux, Marcy-l'Étoile, France), as previously described.[5] In the core

laboratory, a further 200 µL of CSF was inoculated on chocolate agar PolyViteX (bioMérieux) and Columbia agar enriched with a 5% sheep blood (bioMérieux) medium, incubated at 37 °C under 5% CO_2 for five days, and on Columbia agar enriched with 5% sheep blood (bioMérieux) under anaerobic conditions for ten days at 37 °C for bacterial culture and *in-vitro* antibiogram in the standard bacteriology laboratories. For all isolates, antibiograms were validated according to the antibiotic panel approved by the Antibiogram Committee of the French Society for Microbiology (CA-SFM, version V1 2020) (**Appendix 1**).

Installation of the RTM platform in the POC. We updated the equipment available in our POC laboratories by setting up MinION sequencers (Oxford Nanopore Technologies, Oxford, UK) (**Figure 1, Appendix 2**). As an example, in the Marseille POC laboratory, an RTM bench was set up on a surface area of 210 x 70 cm^2, the atmosphere was stabilised at one atm and the temperature was controlled by central air conditioning at 20 °C ± 2 °C. The RTM bench was equipped with a Biocap® hood (Erlab, Val-de-Reuil, France), a clean area for DNA preparation, Qubit® for DNA quantification, a thermal cycler (ThermoFisher, Illkirch, France), an incubator at 20 °C with agitation for the different incubation steps, a vortex for mixing reagents and buffers, magnetic rack, a tube ice rack for enzyme storage during the manipulation, a mini centrifuge at 12,000 g, micro-pipettes with different volumes and a biological waste container (**Figure 1, Appendix 2**). Metagenomic handling was performed in an 1,800 cm^2 workspace. For library sequencing, four MinION instruments were attached in parallel to a powerful computer equipped with at least Windows 10 or a Linux version (16.04 LTS) operating system, an i7 processor, RAM ≥ 8Gb, a USB 3 port, with enough disk space to store the data (~ 1 Tb) (Lenovo, China), and an internet connection. MinION-Sequencer reading, and data storage were

performed using Minknow Oxford Nanopore software version (8.3.1). In addition, Oxford Nanopore EPI2ME software was installed for real-time data analysis.

RTM procedure. Total DNA was extracted from 200 µL of left-over CSF samples using an EZ1 DNA Tissue Kit (Qiagen, Courtaboeuf, France), after 15 minutes of incubation at 56 °C with 20 µL proteinase K (Qiagen), then eluted in a 50 µL volume. For the real-time next-generation sequencing, the Oxford Nanopore library preparation was performed in a 75 µL final volume as previously described.[14] Briefly, 48 µL DNA was prepared and end-repaired in 60 µL containing 3.5 µL of NEBNext FFPE DNA Repair buffer and 3.5 µL of Ultra II End-prep reaction buffer, 2 µL of NEBNext FFPE DNA Repair mix (New England BioLabs, Evry-Courcouronnes, France) and 3 µL of Ultra II End-prep enzyme mix (New England BioLabs). The repair reaction Master Mix was incubated for five minutes at 20 °C followed by a five-minute incubation at 65 °C on a GeneAmp PCR System Thermal Cycler (Applied Biosystems, Foster City, CA, USA). Repaired DNA was purified using equal volumes of Agencourt Ampure XP beads (Beckman Coulter, Villepinte, France), and eluted in 25 µL of sterile water after incubation for five minutes at room temperature and two washes with 70% ethanol. A barcoding step was added to the standard Oxford Nanopore protocol to avoid any cross-contamination and to reduce the cost of the tests. A 22. -µL volume of repaired DNA was barcoded in 50 µL containing 2.5 µL of native barcoding and 25 µL of Blunt/TA Ligase Master Mix (BioLabs) and incubated for ten minutes at room temperature. Barcoded DNA was purified using 50 µL of Agencourt Ampure XP and eluted in 65 µL sterile water, after incubation for five minutes at room temperature and two washes with 70% ethanol. A 65-µL volume of the barcoded DNA was indexed in 100 µL containing 20 µL NEBNext Quick Ligation Reaction Buffer (5X) buffer, 5 µL of Adapter Mix II (AMII) and 10 µL of T4 DNA Ligase and incubated for ten minutes at room temperature. 60 µL of

Agencourt Ampure XP beads were then added to the ligation master mix and incubated for five minutes at room temperature. Two washes were performed using an LFB buffer, then eluted in a 15 µL volume and incubated for ten minutes at room temperature. Finally, 12 µL of the eluted library were added to 37.5 µL sequencing buffer and 25.5 µL loading beads and sequenced for up to two hours on a MinION sequencer (Oxford Nanopore, Oxford Science Park, UK) (**Figure 1**). For rapid pathogen genome identification, the output fastq_pass was generated every 1,500 reads per file to encourage real time analysis, using Minknow specific parameters before starting the sequencing run.

***In-silico* data analysis**

Pathogen genome identification: Pathogen genome sequences were detected in real-time using an in-house pipeline. First, total MinION data were aligned against an in-house database including complete genome sequences for each of *N. meningitidis*, *S. pneumoniae*, *H. influenzae*, *S. agalactiae*, *Escherichia coli*, *Staphylococcus aureus* and *Listeria monocytogenes* (100 sequences per pathogen), VZV (148 sequences), HSV-1 (51 sequences), HSV-2 (33 sequences), Parechovirus (200 sequences) and Enterovirus (300 sequences) using a command line on the IHU server (**Appendix 2**). This analysis was interpreted as positive when the number of sequences for any specific pathogen was ≥ 2. Further, detected sequences were blasted against the NCBI GenBank database to increase the probability of pathogen genome detection using Taxonomer (https://www.taxonomer.com) and Oxford Nanopore EPI2ME online software (**Figure 1, Figure 2**). To confirm real-time identification, quality control of the reads was performed using the FastQC online platform and assembled using the "Canu assembler" tool (Version 2.1.1) on Galaxy Europe online software (https://usegalaxy.eu/). Generated contigs were then aligned using blastn against NCBI GenBank database. The identified hit-blast strain

was used as a reference sequence to map the total MinION reads using CLC Genomics Workbench software version 21.0.3 (Qiagen) with default parameters. Consensus sequences were extracted in FASTA file format for further analysis (**Figure 2**). The *in-silico* prediction of antibiotic resistance-encoding genes was carried out using ResFinder (https://cge.cbs.dtu.dk/services/ResFinder/) and the Resistance Gene Identification (https://card.mcmaster.ca/analyze/rgi) online software, using total pathogen reads and hit-blast strains identified by blastn against the NCBI GenBank database after assembly of the MinION reads, in reference to antibiotics routinely assayed *in-vitro* (**Appendix 1**).

Pathogen genotyping. Microbial genotypes / serotypes were predicted using multi-locus sequence typing tools (MLST) on PubMLST (https://pubmlst.org/organisms), based on total specific sequence data and hit-blast strains identified by blasting against NCBI GenBank database. Virus genotyping was performed directly on the ViPR online database (https://www.viprbrc.org/) (**Figure 2**).

Graphic representation of data. Graphic representation was performed using the R software (version 4.0.3). Pie and doughnut charts were created using the PieDonut function in the webr package (https://www.R-project.org/ and https://CRAN.R-project.org/package=webr).

Cost analysis. We compared estimations of overall cost for the Biofire FilmArray®-based diagnosis of CAM at the POC laboratory with that of RTM-based diagnosis. It should be noted that these estimations incorporated mean cost by sample for RTM (€144) and FilmArray® (€114), based on values calculated with reference prices for materials and reagents in our laboratories. We estimated that 30 RTM assays cost €4,320, including pathogen identification, genotyping, and *in-silico* antibiogram. Meanwhile, we estimated that 30 FilmArray® assays cost €3,411 for pathogen identification only, and this was potentially increased by the cost of the

additional pathogen-specific PCRs, in the case of negative FilmArray® assay, and by additional *in-vitro* investigations for pathogen characterisation. Moreover, the relative cost obviously decreased in cases where a series of CSF samples were tested at the same time. As an example, the cost of RTM decreased to less than €70 / CSF sample for series of 12 samples tested with the same MinION flow-cell, while the cost of FilmArray® was independent of the number of tested CSF samples, being series-insensitive (**Table 3, Appendix 4**).

Role of funding sources. This study was supported by the French Government under the "Investissements d'Avenir "(Investments in the Future) programme managed by the Agence Nationale de la Recherche (ANR, fr: National Agency for Research) [reference: Méditerranée Infection 10-IAHU-03]. This work (lab material and reagents) was supported by the Fondation Méditerranée Infection at the IHU Méditerranée Infection and bacteriology and hygiene laboratory at Nîmes University Hospital. Funders played no role in the study design, data collection, data analyses, interpretation, or writing of the report.

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RESULTS

General data. CSF samples collected from 52 patients prospectively investigated in this study included 24 CSF samples from the IHU Méditerranée Infection POC laboratory in Marseille and 28 CSF samples from the bacteriology and hygiene laboratory at Nîmes University Hospital. These 52 CSF samples were collected from 24 female patients and 28 male patients, aged between 0 and 90 years (median, 38 years old), investigated between December 2020 and July 2021 (**Table 1**, **Appendix 3**).

Routine investigations. Routine FilmArray® assays detected a microorganism in 49 CSF samples. Bacterial pathogens found in 45 CSF samples included 21 *S. pneumoniae*, nine *N. meningitidis*, eight *H. influenzae*, three *S. agalactiae*, two *L. monocytogenes* and one each of *E. coli* and *S. aureus*. In addition, three *Herpesvirus*-1 and one *Varicella-zoster virus* (VZV) were detected. All viral cases resulted from PCR, while 20 bacterial meningitis cases were confirmed by culture and RT-PCR, 12 by RT-PCR only, including one case (sample 31) of *S. pneumoniae* > 35 Ct which was interpreted as negative in routine POC diagnosis, 12 cases with FilmArray® only, and PCR and culture failed to identify one case of *H. influenzae* (sample 20). The 20 culture positive CSFs grew 12 *S. pneumoniae*, three *N. meningitidis*, two *H. influenzae*, two *L. monocytogenes* and one *S. aureus,* while the other 26 CSFs detected positive for bacteria by RT-PCR were culture-negative. *In-vitro* antibiotic investigation yielded 15 susceptible bacteria (nine *S. pneumoniae*, two each of *N. meningitidis* and *H. influenzae*, one each of *L. monocytogenes* and *S. aureus*), three *S. pneumoniae* which were resistant to erythromycin, clindamycin, pristinamycin, doxycycline, one *N. meningitidis* which was resistant to amoxicillin and rifampicin, and one *L. monocytogenes* which was resistant to trimethoprim and clindamycin (**Table 1**).

RTM investigations. In total, RTM detected pathogen genomes in 47 leftover CSF samples. Bacteria detected in 43 CSF samples included 20 *S. pneumoniae*, nine *N. meningitidis*, seven *H. influenzae*, three *S. agalactiae*, two *L. monocytogenes*, and one each of *E. coli* and *Staphylococcus aureus.* Viral pathogens detected in four CSF samples included three *Herpesvirus-1* and one Varicella-Zoster Virus. In addition, RTM yielded five negative CSF samples. In 70.2% of CSF samples, pathogen genome detection in a 20-minute sequencing run (**Figure 3**, **Table 2**) yielded 16 *S. pneumoniae*, seven *N. meningitidis*, six *H. influenzae*, one *S.*

agalactiae and one VZV (median number of reads = 23), resulting from a blast analysis of MinION data against the in-house database and EPI2ME online analysis. A total of 47/52 CSF samples were detected as positive after two hours (median number of reads of 456.5). Genomic data analysis showed 19/47 (40.4%) of positive cases with >50% genome coverage including eight *S. pneumoniae*, five *N. meningitidis*, four *H. influenzae*, one each of *S. agalactiae* and VZV (**Figure 1**, **Table 2**). Viral cases were identified directly by blast against the in-house database and EPI2ME online software and confirmed by specific RT-PCR. The false negative *S. pneumoniae* (sample 31) was confirmed by negative Illumina pair-end metagenomics, as previously described,[16] may be due to failed DNA extraction and/or the limitation of RTM to detect low pathogen levels in the CSF (>35 Ct). The *in-silico* antibiogram analysis yielded 30/43 susceptible bacteria, 12 resistant bacteria and was not carried out in one *E. coli* strain due to the poor quality of the sequence generated by MinION (**Table 1**).

Comparison between RTM and routine investigations. Two discordances were noted by comparing RTM and routine investigation data. One case of *H. influenzae* (sample 20) detected by FilmArray® but not by RTM was eventually interpreted as a false-positive of RT-PCR,[5] in agreement with a negative specific PCR and culture. One case of *S. pneumoniae* (sample 31) was detected by RT-PCR but not by RTM. Further controls by routine RT-PCR yielded a >35 Ct and the culture remained negative. A total of 23/43 (53.5%) positive cases of bacterial meningitis diagnosed by BioFire FilmArray® failed in culture, and it was not possible to carry out an *in-vitro* antibiogram (**Table 1**). From the 16 susceptible bacteria which were identified in routine bacteriology, antibiotic susceptibility testing of cultured bacteria yielded 12/16 concordant *in-vitro* and *in-silico* antibiograms, while three bacteria (*H. influenzae*, *S. pneumoniae* and *S. aureus*) were *in-silico* resistant for beta-lactamins, macrolides, doxycycline, fluoroquinolone,

aminoglycoside and chloramphenicol (**Table 1**), probably due to the absence of expression of resistance despite the possession of the antibiotic resistance encoding genes. A discordance in antibiogram was observed in one *L. monocytogenes* which was *in-vitro* resistant to trimethoprim and clindamycin, being *in-silico* resistant to fosfomycin. We concluded that this discordance was due to the low quantity of genomic data to identify all antibiotic-encoding genes, as well as the hit-blast strain for further analysis. Furthermore, partial discordance was observed in samples 1 and 2, identified an *in-vitro* resistant *S. pneumoniae* for trimethoprim and streptogramin b, while sample 1 was *in-silico* resistant to lincomycin, quinupristin, pristinamycin, rifamycin, fluoroquinolone, aminoglycoside and phenicol, and *in-silico* resistant to lincomycin and phenicol in sample 2 (**Table 1**). In addition, an *in-silico* antibiogram was successfully performed in 22 uncultured bacteria, including 18 susceptible bacteria (seven *S. pneumoniae*, five *N. meningitidis,* four *H. influenzae*, and two *S. agalactiae*), one *N. meningitidis* was *in-silico* predicted to be resistant to penicillin A and rifampicin, which was due to the presence of the *farB* gene encoding for the efflux pump involved in rifampicin resistance; one *S. agalactiae* was *in-silico* predicted to be resistant to erythromycin, azithromycin and spiramycin; one *H. influenzae in-silico* predicted to be resistant for amoxicillin, ampicillin, cephalothin, piperacillin, ticarcillin and one *S. pneumoniae in-silico* predicted resistant for erythromycin, streptogramin B, chloramphenicol and lincomycin. In addition, one *L. monocytogenes* which was *in-vitro* resistant to trimethoprim and clindamycin was *in-silico* resistant for fosfomycin. This may be due to the low quantity of genomic data to identify the hit-blast strain and antibiogram analysis.

Bacterial genotyping: Genome-sequence-derived-MLST analysis yielded 14 bacterial serotypes derived from 40 MLST profiles. Twenty genotyped *S. pneumoniae* yielded six serotypes (seven 3A serotype, five 11A, four 6B, two 16F, one 5A and one 12F serotype), three *N. meningitidis*

serotypes (5/9 B serotype, 3/9 C serotype and 1/9 A serotype), three *H. influenzae* serotypes (3/6 B serotype, 2/6 F serotype and 1/6 non-typable), three cases of *S. agalactiae* V serotype and only one *L. monocytogenes* 3d serotype (**Figure 4**, **Table 2**). In addition, 50% of *S. pneumoniae* positive patients were over the age of 55, with a dominance of 3A and 6B serotypes. Six out of nine *N. meningitidis* cases were aged 30 or under, with a dominance of the C-serotype. Four out of seven *H. influenzae* cases were aged 25 or under and three were over the age of 45. The b-serotype was identified in patients aged 0, 45 and 65 years old, the F-serotype was identified in two patients aged 25 and 90 years old. Two non-typable *H. influenzae* cases were identified in patients below the age of two, and one *L. monocytogenes* 3d serotype was identified in a patient over the age of 80. There was no association between bacteria serotypes, age, and gender of the patients. According to the low number of viral cases diagnosed in this series, virus genotyping was not performed.

Virology data. Four viral infections were diagnosed in immunocompetent patients in this series (**Table 1**). Only DNA viruses were detected here, including three reactivated HSV-1 (samples 21, 22, 23), diagnosed in women aged 14 and 77 and one 68-year-old man, all of whom were diagnosed with meningoencephalitis. In addition, wild-type VZV (https://www.viprbrc.org/) was detected in only one CSF (sample 24), collected from n apparently immunocompetent 29-year-old woman with a past medical history of childhood VZV infection, no further recent contact with the virus, including no vaccination, and no clinical diagnosis of zoster; all data are suggestive of VZV-reactivation (**Table 2**, **Figure 4**).

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DISCUSSION

We used RTM, directly applied to leftover CSF samples, to investigate the diagnosis of community-acquired meningitis at the POC laboratories in two university hospitals in southern France. The multiplex RT-PCR assays which are currently used routinely in the POC laboratory for this purpose only detect pieces of the pathogen genome, providing detection and identification.[5] This study indicated that, in addition to detection and identification, an RTM diagnostic strategy using Oxford Nanopore sequencing performed well on the diagnosis of known and non-routinely detectable pathogens in CSF samples, the antibiotic susceptibility profile, as well as their genotype.[14,16,19] Moreover, cross-contamination, which has been shown to limit the interpretation of positive multiplex assay results in several original studies and resulting meta-analyses,[5,20,21] was removed by the addition of a barcoding step; as illustrated here in one case of false-positive BioFire filmArray® *H. influenzae*.

In contrast to multiplex RT-PCR approaches which require specific conditions and equipment,[5,6,22] RTM can be implemented in a surface area of less than two square metres in a POC laboratory using simple materials and with no requirement for advanced bioinformatics knowledge. This makes RTM a useful POC diagnostic tool, based on the simplicity, rapidity and cost-effectiveness of the process.[15,23,24] Additional pieces of information were added concerning bacterial genotype/serotype, which are not applicable using conventional methods limited by the pathogen genome detection and culture, failing in 22/43 CSF, which required a specific PCR target for all pathogens.[5,6] Bacteria profiling by *in-silico* investigation enabled the detection of the presence of genes encoding for antibiotic resistance, which was concordant in 12/16 (75%) of cultured CSFs with the *in-vitro* investigation. In addition, RTM detected the presence of genes encoding for antibiotic resistance further phenotypically detected in three culture-positive CSFs and four additional failed bacteria cultures, despite the low level of pathogens in the CSF.

Supplementary information due to the mechanism of antibiotic resistance was added by *in-silico* analysis in one case of *N. meningitidis* which was *in-vitro* anticipated to be rifampicin-resistant after *in-silico* detection of the *farB* gene encoding for efflux pumps.[25] In addition, we found significant pathogen genotype diversity, mostly represented by *S. pneumoniae* 3A, 11A and 6B serotypes, followed by *N. meningitidis* B and C-serotypes. The *H. influenzae* b-serotype was identified most often in this study, followed by the non-typable and *H. influenzae* A serotype. This enabled the real-time genomic fine and accurate surveillance of bacteria genotypes and variants circulating in southern France, based on pathogen genome sequences (**Figure 4**), for the definition of a new strategy of infectious disease control including vaccination, as previously described in a case of non-typable *H. influenzae* meningitis identified in a patient vaccinated with the b-serotype.[14] In addition, this strategy successfully detected four wild type viral DNA samples in agreement with routine multiplex RT-PCR,[26] validating its application for the direct investigation of DNA pathogens.

The limits we encountered reflect ways in which the method can be improved. The failure of RTM in one CSF (sample 31) which was detected positive in routine multiplex-RT-PCR indicated the need to increase RTM sensitivity for RT-PCR-detected pathogens with Ct > 35, given the higher sensitivity of the BioFire FilmArray® assay based on nested multiplex-RT-PCR.[5,7] Increased sensitivity could be achieved by improving DNA extraction through an adapted automatic library preparation protocol for low pathogen levels, including microbial genome enrichment and human genome depletion.[7,23,27] Also, the enlargement of the microbial panel included in the in-house database and its combination into one protocol DNA and RNA RTM is needed. This enlargement would allow for the one-shot detection of most pathogens responsible for community-acquired meningitis and meningoencephalitis, especially RNA

viruses, the most frequently encountered causative agents of meningitis[28] and non-routinely diagnosed bacterial meningitis at the POC laboratory.[19]

CONCLUSION

This study goes beyond a few previous reports[14,16,19] which all indicated that RTM has the potential to complement current multiplex RT-PCR assays for the rapid detection, genotyping and *in silico* antibiotic resistance profiling of pathogens responsible for community-acquired meningitis. This technique is already competitive in terms of time with the routine multiplex-based diagnostics in POC laboratories. Implementation of RTM as a POC diagnostic tool for life-threatening meningitis may provide real-time genomic surveillance of meningitis causative pathogen variants circulating in the study area, in order to define a new strategy of epidemiological control and vaccination. The authors are working towards implementing RTM in routine POC in selected situations, including potential multiplex-PCR failures, based on the diagnostic results reported in this study, along with a preliminary cost analysis and preliminary training course for residents in medical biology (**Table 3, Appendix 4**). Further developments may include the application of RTM to cases of undocumented meningitis and RNA virus cases to enrich the repertoire of meningitis-causative pathogens, non-routinely diagnosed in CNS diseases.

Contributors

MM was responsible for the experimental design, implementation of the RTM workbench at the POC, software, creation of the database, bioinformatic data analysis, interpretation and writing the original draft paper. QK ensured sample collection, helped with the implementation of the RTM workbench at the POC, and data analysis and writing. AB and RS were responsible for sample collection and clinical data. FS carried out statistical analysis. CR and LH were responsible for conceptualisation, reagents, implementation of the RTM workbench at the POC and validation. JPL and MD were involved in writing the original draft paper, critically reviewing the paper, validation, management of the work, and funding.

All authors read and approved the final version of the manuscript

Declaration of interest

The authors declare no conflicts of interest. In particular, the authors did not receive any contribution from any of the suppliers mentioned in this report.

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Data Sharing Statement

All the extracted scaffold and contigs FASTA sequences corresponding to different pathogens were submitted to GenBank NCBI, available through BioProject No. PRJEB49201.

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Figure Legends

Figure 1: CSF workflow for the diagnosis of community-acquired meningitis in the POC laboratory for the prospective investigation of the 52 CSF series. When the CSF sample was received at the POC laboratory, several tests were performed to detect the meningitis causative agent. **a**) Systematically, the emergency multiplex BioFire FilmArray® assay was performed using 200 µL CSF when the sample was received, followed by quantification of the blood cells. **b**) As per routine diagnosis, all CSF samples received at the POC were routinely cultured. **c**) The RTM diagnosis was performed in a total workflow that did not exceed four hours. Sample preparation and DNA extraction from leftover CSF samples took 35 minutes, Oxford Nanopore library preparation took 65 minutes, and the MinION library sequencing took two hours. **d**) Real-time genome identification was performed directly by blast of the MinION generated data against an in-house database, then against the NCBI database using EPI2ME and Taxonomer online software. **e**) Antibiotic resistance and pathogen genotyping were *in-silico* predicted using the ResFinder online database (https://cge.cbs.dtu.dk/services/ResFinder-4.1/), and the MLST online database (https://pubmlst.org/organisms).

Abbreviations: CSF: cerebrospinal fluid. WBC: white blood cell. RBC: red blood cell. RTM: real-time metagenomics. MLST: Multi-Locus Sequence Typing.

Figure 2: Bioinformatic pipeline. Rapid pathogen genome identification performed by direct alignment of MinION reads with an in-house database, further blasted against NCBI GenBank using Taxonomer and EPI2ME online software. The analysis was interpreted as positive when ≥ 2 pathogen-specific reads were identified. To confirm pathogen identification, the quality of

MinION reads was controlled by FastQC before assembly by "Canu assembler" on Galaxy Europe online software (https://usegalaxy.eu/). Hit-blast strains were identified by blastn of the generated contigs against GenBank database, then used as reference genome for mapping the total MinION reads by CLC Genomics Workbench software version 21.0.3 (Qiagen). The consensus genomes were extracted in FASTA files for pathogens genotyping on MLST on PubMLST (https://pubmlst.org/organisms) for bacteria genotyping and on the ViPR online database (https://www.viprbrc.org/) for virus genotyping. The *in-silico* antibiogram was predicted on ResFinder (https://cge.cbs.dtu.dk/services/ResFinder/) and Resistance Gene Identification (https://card.mcmaster.ca/analyze/rgi) platforms using the total MinION reads and the generated FASTA sequences with default settings.

Figure 3: Real-time metagenomic data analysis and pathogen characterisation. **a**) Real-time data analysis and pathogen identification after 20 minutes of sequencing. Thirty-one CSF samples were diagnosed as positive after a 20-minute sequencing run, including 16 *S. pneumoniae*, seven *N. meningitidis*, six *H. influenzae*, one *S. agalactiae,* and one VZV. **b**) Total generated data and pathogen identification after a two-hour sequencing run. A total of 47/52 CSFs were diagnosed as positive after two hours RTM, included 20 *S. pneumoniae*, nine *N. meningitidis*, seven *H. influenzae*, three *S. agalactiae*, three *Herpes Simplex Virus*, two *L. monocytogenes*, one case each of *E. coli* and *S. aureus*, and one *VZV*.

Figure 4: Pathogen genotyping and distribution according to the causative bacteria. Genotype investigation was performed on the PubMLST database (https://pubmlst.org/) for bacteria pathogens and the ViPR database (https://www.viprbrc.org/) for virus genotyping. A total of 14

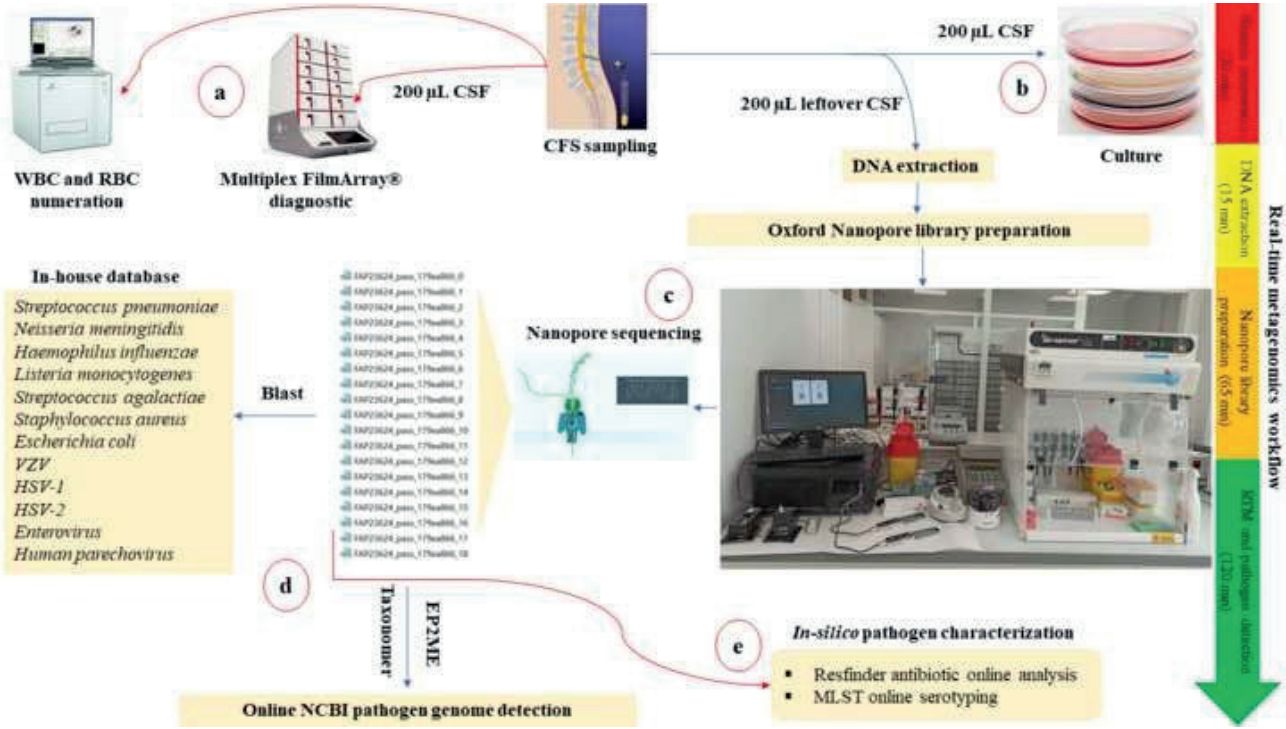
555 bacterial serotypes were identified from 40 MLST profiles, 20 *S. pneumoniae* serotype (seven
556 3A serotype, five 11A, four 6B, two 16F, one 5A and one 12F serotype), three *N. meningitidis*
557 serotypes (5/9 B serotype, 3/9 C serotype and 1/9 A serotype), three *H. influenzae* serotypes (3/6
558 B serotype, 2/6 F serotype and 1/6 non-typable), three *S. agalactiae* V serotype, and one *L.*
559 *monocytogenes* 3d serotype. All viruses detected in this series were for an unknown serotype.

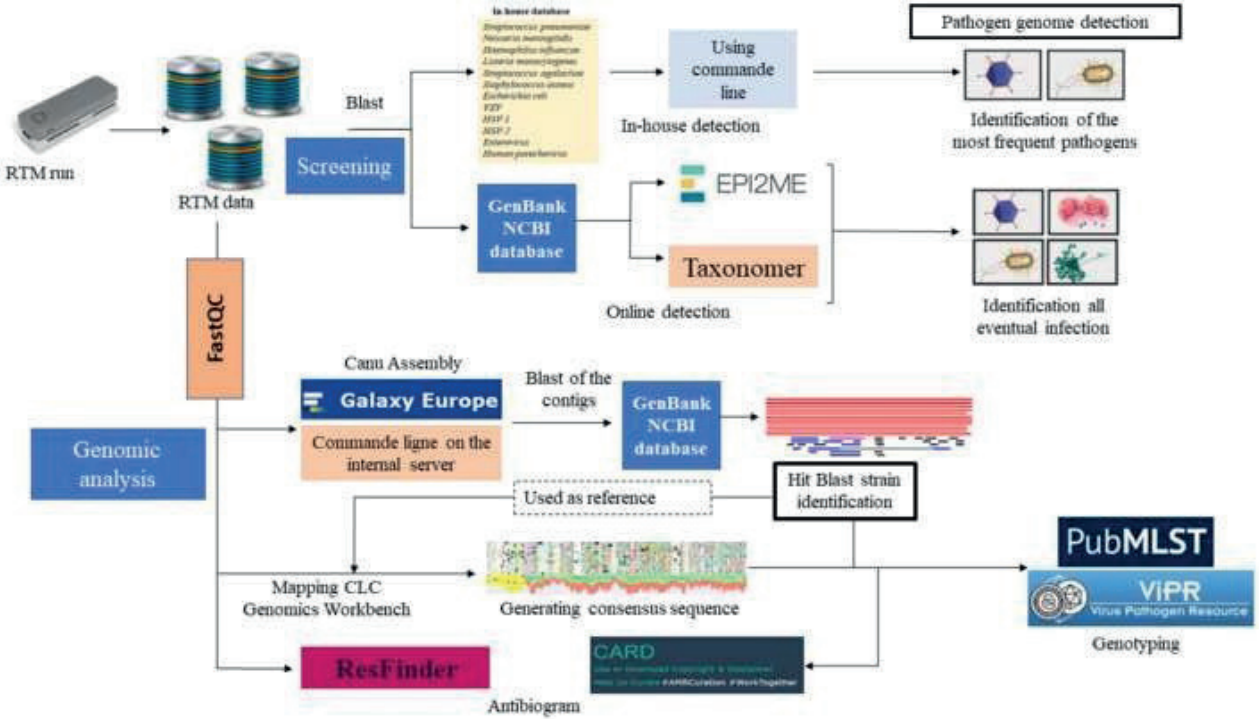
Table legends

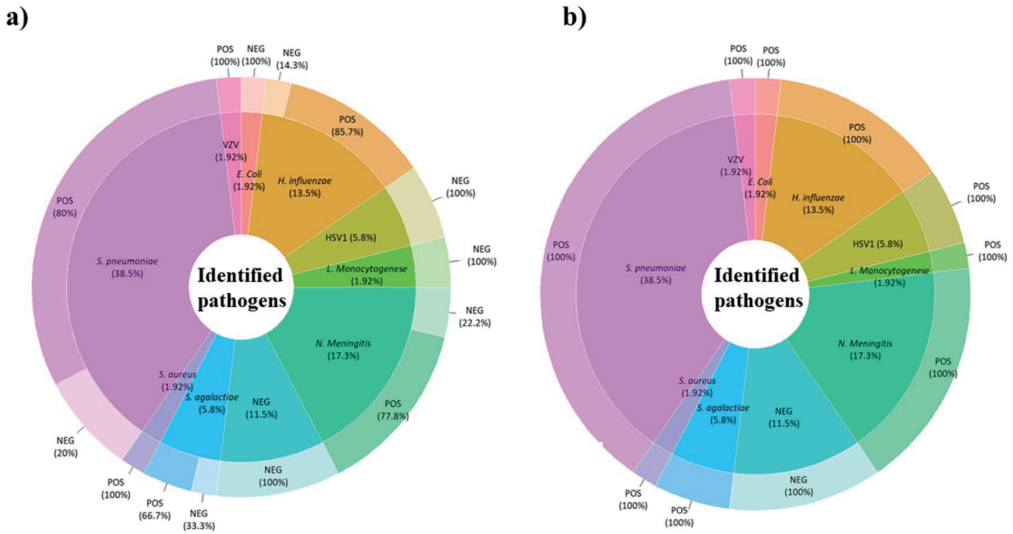
Table 1: Concordance and discordance of routine diagnosis with RTM, *in-vitro* and *in-silico* antibiogram.

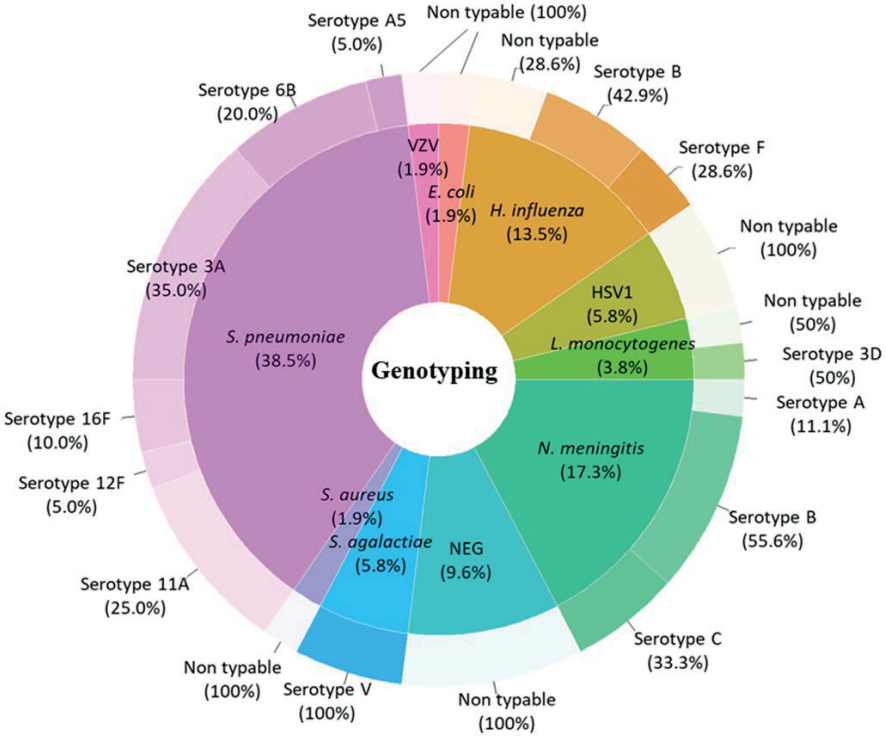
Table 2: partial and total genomic data, RTM analysis and bacteria genotyping.

Table 3: Cost analysis and comparison of RTM and routine FilmArray® test.









Patient	Age	Gender	Final diagnostic	Biofire FilmArray	MinION	Culture	Specific RT-PCR	*In-vitro* antibiogram	*In-silico* antibiogram
Sample 1	90	M	*Streptococcus pneumoniae*	Positive	Positive	Positive	Not realized	Macrolides and related groups (erythromycin, clindamycin, streptogramin b), tetracycline, trimethoprim	Macrolides and related groups (erythromycin, clindamycin, lincomycin, quinupristin, pristinamycine, virginiamycin), tetracycline, rifamycin, fluoroquinolone, aminoglycoside, phenicol
Sample 2	70	M	*Streptococcus pneumoniae*	Positive	Positive	Positive	Not realized	Macrolides and related groups (erythromycin, clindamycin, streptogramin b), tetracycline, trimethoprim	Macrolides and related groups (erythromycin, clindamycin, lincomycin), tetracycline, phenicol
Sample 3	25	M	*Neisseria meningitidis*	Positive	Positive	Positive	Not realized	Susceptible	Susceptible
Sample 4	80	M	*Streptococcus pneumoniae*	Positive	Positive	Negative	Not realized	Not realized	Susceptible
Sample 5	84	M	*Listeria monocytogenes*	Positive	Positive	Positive	Not realized	**Clindamycin, Trimethoprim**	**Fosfomycin**
Sample 6	66	F	*Streptococcus pneumoniae*	Positive	Positive	Positive	Not realized	Macrolides and related groups (erythromycin, clindamycin, streptogramin b), tetracycline	Macrolides and related groups (erythromycin, clindamycin, streptogramin b quinupristin, pristinamycine, virginiamycin), tetracycline
Sample 7	65	F	*Haemophilus influenzae*	Positive	Positive	Positive	Not realized	Susceptible	Amoxicillin/ampicillin, piperacillin, ticarcillin, cephalothin
Sample 8	64	F	*Neisseria meningitidis*	Positive	Positive	Negative	Not realized	Not realized	Susceptible
Sample 9	30	F	*Streptococcus pneumoniae*	Positive	Positive	Positive	Not realized	Susceptible	Susceptible
Sample 10	57	F	*Streptococcus pneumoniae*	Positive	Positive	Negative	Not realized	Not realized	Susceptible
Sample 11	2	M	*Streptococcus pneumoniae*	Positive	Positive	Positive	Not realized	Susceptible	Susceptible
Sample 12	45	M	*Streptococcus*	Positive	Positive	Negative	Not realized	Not realized	Susceptible

			pneumoniae						
Sample 13	0	F	*Streptococcus agalactiae*	Positive	Positive	Negative	Not realized	Not realized	Macrolides and related groups (erythromycin, spiramycin, azithromycin)
Sample 14	7	F	*Neisseria meningitidis*	Positive	Positive	Negative	Not realized	Not realized	Susceptible
Sample 15	58	M	*Neisseria meningitidis*	Positive	Positive	Negative	Not realized	Not realized	Susceptible
Sample 16	0	F	*Streptococcus agalactiae*	Positive	Positive	Negative	Not realized	Not realized	Susceptible
Sample 17	33	M	*Streptococcus pneumoniae*	Positive	Positive	Positive	Not realized	Susceptible	Macrolides and related groups (erythromycin, clindamycin, streptogramin b quinupristin, pristinamycine, virginiamycin)
Sample 18	0	M	*Haemophilus influenzae*	Positive	Positive	Negative	Not realized	Not realized	Amoxicillin/ampicillin, piperacillin, ticarcillin, cephalothin
Sample 19	32	M	**Negative**	**Negative**	**Negative**	**Negative**	**Not realized**	**Not realized**	**Not realized**
Sample 20	28	F	**Negative**	*Haemophilus influenzae*	**Negative**	**Negative**	**Negative**	**Not realized**	**Not realized**
Sample 21	68	M	*Herpes Simplex Virus 1*	Positive	Positive	Negative	Positive	Not realized	Not realized
Sample 22	14	F	*Herpes Simplex Virus 1*	Positive	Positive	Negative	Positive	Not realized	Not realized
Sample 23	77	F	*Herpes Simplex Virus 1*	Positive	Positive	Negative	Positive	Not realized	Not realized
Sample 24	29	F	*Varicella Zoster Virus*	Positive	Positive	Negative	Positive	Not realized	Not realized
Sample 25	89	F	*Haemophilus influenzae*	Positive	Positive	Negative	Not realized	Not realized	Susceptible
Sample 26	74	F	*Streptococcus pneumoniae*	Positive	Positive	Negative	Not realized	Not realized	Macrolides and related groups (erythromycin, clindamycin, streptogramin b), phenicol
Sample 27	49	F	*Haemophilus influenzae*	Positive	Positive	Negative	Not realized	Not realized	Susceptible

Sample 28	22	M	*Listeria monocytogenese*	Positive	Positive	Positive	Not realized	Susceptible	Susceptible
Sample 29	0	M	*Streptococcus pneumoniae*	Positive	Positive	Positive	Positive	Susceptible	Susceptible
Sample 30	78	F	*Streptococcus pneumoniae*	Positive	Positive	Negative	Positive	Not realized	Susceptible
Sample 31	11	M	*Streptococcus pneumoniae*	Positive	Negative	Negative	Positive>35Ct	Negative	Not realized
Sample 32	22	M	*Neisseria meningitidis*	Positive	Positive	Negative	Positive	Not realized	Penicillin A
Sample 33	18	M	*Neisseria meningitidis*	Positive	Positive	Negative	Positive	Not realized	Susceptible
Sample 34	42	M	*Neisseria meningitidis*	Positive	Positive	Negative	Positive	Not realized	Susceptible
Sample 35	0	M	*Haemophilus influenzae*	Positive	Positive	Positive	Positive	Susceptible	Susceptible
Sample 36	0	M	*Haemophilus influenzae*	Positive	Positive	Negative	Positive	Not realized	Susceptible
Sample 37	40	M	*Haemophilus influenzae*	Positive	Positive	Negative	Positive	Not realized	Susceptible
Sample 38	57	M	*Staphylococcus aureus*	Positive	Positive	Positive	Positive	Susceptible	Cefoxitin (*mecA*)
Sample 39	40	M	**Negative**	**Negative**	**Negative**	**Negative**	**Negative**	**Not realized**	**Not realized**
Sample 40	59	F	*Streptococcus pneumoniae*	Positive	Positive	Negative	Positive	Not realized	Susceptible
Sample 41	33	F	*Neisseria meningitidis*	Positive	Positive	Positive	Positive	Susceptible	Susceptible
Sample 42	18	F	*Streptococcus agalactiae*	Positive	Positive	Negative	Positive	Not realized	Susceptible
Sample 43	0	M	*Streptococcus pneumoniae*	Positive	Positive	Positive	Positive	Susceptible	Susceptible
Sample 44	20	F	*Neisseria meningitidis*	Positive	Positive	Positive	Positive	Susceptible	Penicillin A
Sample 45	23	F	**Negative**	**Negative**	**Negative**	**Negative**	**Negative**	**Not realized**	**Not realized**
Sample 46	51	M	*Streptococcus pneumoniae*	Positive	Positive	Positive	Positive	Susceptible	Susceptible
Sample 47	55	F	*Streptococcus*	Positive	Positive	Negative	Positive	Not realized	Susceptible

			pneumoniae						
Sample 48	64	F	*Streptococcus pneumoniae*	Positive	Positive	Positive	Positive	Susceptible	Susceptible
Sample 49	0	M	*Escherichia coli*	Positive	Positive	Negative	Positive	Not realized	Not realized
Sample 50	6	M	*Streptococcus pneumoniae*	Positive	Positive	Negative	Positive	Not realized	Susceptible
Sample 51	85	F	*Streptococcus pneumoniae*	Positive	Positive	Positive	Positive	Susceptible	Susceptible
Sample 52	72	M	*Streptococcus pneumoniae*	Positive	Positive	Positive	Positive	Susceptible	Susceptible

Samples	Run time	Identied pathogen	Specific reads at 20 minutes	Total reads at 2 hours	Specific reads at 2 hours	Specific reads (%)	Number of nucleotide	Genome coverage	Genotyping
Sample 1	2H	*Streptococcus pneumoniae*	5897	174,156	33,465	19.22	1,940,109	95.17	Serotype 16F
Sample 2	2H	*Streptococcus pneumoniae*	7457	125,520	35,055	27.93	1,959,603	87.48	Serotype 6B
Sample 3	2H	*Neisseria meningitidis*	45	116 ,752	905	0.78	762,285	34.01	Serotype B
Sample 4	2H	*Streptococcus pneumoniae*	0	9,137	6	0.065667068	4,183	0.02	Serotype 3A
Sample 5	2H	*Listeria monocytogenes*	0	480,000	21	0.00004375	14,597	0.5	Serotype 3d
Sample 6	2H	*Streptococcus pneumoniae*	7	151,187	135	0.089293392	73,893	3.36	Serotype 11A
Sample 7	2H	*Haemophilus influenzae*	3471	180,169	26,014	14.43866592	1,751,169	88.37	Serotype b
Sample 8	2H	*Neisseria meningitidis*	0	3,830	3	0.078328982	2,687	0.1	Serotype C
Sample 9	2H	*Streptococcus pneumoniae*	0	34,860	12	0.034423408	4,578	0.22	Srotype 16F
Sample 10	2H	*Streptococcus pneumoniae*	5	11,260	132	1.172291297	65,775	0.3	Serotype A5
Sample 11	2H	*Streptococcus pneumoniae*	3451	30,015	17,074	56.88489089	1,994,683	93.65	Serotype 3A
Sample 12	2H	*Streptococcus pneumoniae*	245	131,943	1,652	1.252055812	1,346,254	63.2	Serotype 3A
Sample 13	2H	*Streptococcus agalactiae*	123	206,130	1,017	0.493377965	390,297	19.38	Serotype V
Sample 14	2H	*Neisseria meningitidis*	4513	60,134	18,965	31.53789869	2,023,530	93.96	Serotype C
Sample 15	2H	*Neisseria meningitidis*	411	57,227	3,713	6.488196131	1,644,843	73.43	Serotype A
Sample 16	2H	*Streptococcus agalactiae*	341	125,815	2,058	1.635735008	1,504,297	71.3	Serotype V
Sample 17	2H	*Streptococcus pneumoniae*	239	6,501	1,175	18.07414244	517,575	24.3	Serotype 11A
Sample 18	2H	*Haemophilus influenzae*	314	9,283	1,406	15.14596574	1,318,171	66.52	Serotype b
Sample 19	2H	Negative	0	39,810	0	0	0	0	Not realised
Sample 20	2H	Negative	0	8,110	0	0	0	0	Not realised
Sample 21	2H	*Herpes Simplex Virus 1*	0	4,760	5	0.105042017	501	0.03	Unkown
Sample 22	2H	*Herpes Simplex Virus 1*	0	14,110	2	0.014174344	271	0.02	Unkown
Sample 23	2H	*Herpes Simplex Virus 1*	0	7,082	9	0.127082745	1,519	0.99	Unkown
Sample 24	2H	*Varicella Zoster Virus*	41	251,411	608	0.241835083	86,736	69.38	Unkown
Sample 25	2H	*Haemophilus influenzae*	967	775,438	4,352	0.005612312	1,555,463	83.8	Serotype f
Sample 26	2H	*Streptococcus pneumoniae*	2	74,278	31	0.000417351	18,086	0.8	Serotype 6B

Sample 27	2H	*Haemophilus influenzae*	8	14,994	63	0.004201681	2,8751	1.45	Serotype f
Sample 28	2H	*Listeria monocytogenese*	0	511,274	6	1.17354E-05	9,104	0.03	Not realised
Sample 29	2H	*Streptococcus pneumoniae*	29	305,696	2,566	0.00839396	1,138,209	51.8	Serotype 6B
Sample 30	2H	*Streptococcus pneumoniae*	46	52,353	136	0.00259775	44,612	3	Serotype 3A
Sample 31	2H	Negative	0	52,000	0	0	0	0	Not realised
Sample 32	2H	*Neisseria meningitidis*	63	64,276	1,107	0.017222603	1,918,857	85.6	Serotype B
Sample 33	2H	*Neisseria meningitidis*	51	92,000	591	0.006423913	796,395	37.12	Serotype C
Sample 34	2H	*Neisseria meningitidis*	4	228,118	238	0.00104332	359,034	16.73	Serotype B
Sample 35	2H	*Haemophilus influenzae*	192	84,000	2,751	0.03275	1,869,540	99.06	Non Typable
Sample 36	2H	*Haemophilus influenzae*	23	287,552	561	0.001950951	378,275	20.66	Serotype b
Sample 37	2H	*Haemophilus influenzae*	0	69,418	5	7.20274E-05	1282	0.06	Non Typable
Sample 38	2H	*Staphylococcus aureus*	24	288,211	477	0.001655037	219011	7.81	Not realised
Sample 39	2H	Negative	0	60,069	0	0	0	0	Not realised
Sample 40	2H	*Streptococcus pneumoniae*	97	88,376	1,125	0.0127297	620,988	29.15	Serotype 3A
Sample 41	2H	*Neisseria meningitidis*	356	91,198	8,726	0.095681923	2,054,566	91.29	Serotype B
Sample 42	2H	*Streptococcus agalactiae*	0	7,409	5	0.000674855	573	0	Serotype V
Sample 43	2H	*Streptococcus pneumoniae*	0	74,260	8	0.00010773	27,083	1.3	Serotype 12F
Sample 44	2H	*Neisseria meningitidis*	0	7,160	1,638	0.22877095	1,161,716	51.16	Serotype B
Sample 45	2H	Negative	Negative	82,070	0	0	0	0	Not realised
Sample 46	2H	*Streptococcus pneumoniae*	562	199,270	6,047	0.030345762	2,064,033	96.9	Serotype 11A
Sample 47	2H	*Streptococcus pneumoniae*	12	4,238	456	0.107597924	1,011,157	47.5	Serotype 3A
Sample 48	2H	*Streptococcus pneumoniae*	4,358	163,052	82,665	0.506985502	2,081,073	97.7	Serotype 11A
Sample 49	2H	*Escherichia coli*	0	2,950	2	0.000677966	/	/	Not realised
Sample 50	2H	*Streptococcus pneumoniae*	2	12,370	14	0.00113177	4,009	0.02	Serotype 6B
Sample 51	2H	*Streptococcus pneumoniae*	0	3,221	2,094	0.650108662	783,321	36.8	Serotype 11A
Sample 52	2H	*Streptococcus pneumoniae*	435	82,070	7,632	0.092993786	2,028,482	95.23	Serotype 3A

Table 3.

Approach	Blunt/TA Ligase Master Mix	NEBNext® FFPE DNA Repair Mix	NEBNext® FFPE DNA Repair Mix	NEBNext® Quick Ligation™*	Flow Cell (R9)	Ligation Kit	Flow Cell Priming Kit*	Flow Cell Wash Kit	Native Barcoding (PCR-free) *	Biofire FilmArray cardridge	Veritable cost by test
RTM	1,44	6 €	9 €	12.72	54	45 €	5 €	13 €	11 €	0 €	143,3€

Click here to access/download
Supplemental Data
Appendix 6.xlsx

Conclusions et perspectives

En conclusion, ce travail de Thèse, portant sur le développement expérimental et l'utilisation de la métagénomique directe du liquide céphalorachidien (LCR) prélevé pour réaliser le diagnostic des méningites infectieuses, a été supporté par une revue de la littérature sur ce sujet, qui a guidé nos travaux expérimentaux. Guidant également nos travaux expérimentaux, notre analyse rétrospective de 20.779 LCR analysés dans les laboratoires de microbiologie de Nîmes et de Marseille entre décembre 2014 et décembre 2019, a montré pour la première fois l'existence d'une dynamique géographique des épidémies de syndrome méningé communautaire, dynamique Ouest-Est dans cette région du Sud-Est de la France, et sa corrélation avec les variations de la température atmosphérique, alors même que 89,4% des cas de syndrome méningé communautaire restent non documentés. Basé sur ces deux travaux de revue, nous avons développé deux stratégies expérimentales permettant le suivi génomique des méningites à Entérovirus par métagénomique NGS directe du LCR, stratégies que nous avons adaptées à la pratique diagnostique des laboratoires point-de-soins, par utilisation de la technologie Nanopore, pour la détection, le génotype et le profil de sensibilité aux antimicrobiens du pathogène directement à partir de sa séquence génomique. L'évaluation de ce travail expérimental contributif prospectif réalisé dans le cadre de cette Thèse a été valorisé par plusieurs publications scientifiques illustrant l'application de ce protocole pour le diagnostic microbiologique de la méningite communautaire par comparaison à la RT-PCR multiplexe, au laboratoire point-de-soins.

Une première ligne de perspectives offerte par notre travail de Thèse, nous avons identifié des points d'amélioration qu'il conviendrait de mettre en œuvre en perspectives à nos travaux de Thèse. Techniquement il convient de faire évoluer nos protocoles afin de dépléter le LCR du génome humain afin de récupérer suffisamment de données génomiques du pathogène pour la suite des analyses *in-silico*. Également, une optimisation de coût moyen et la standardisation d'un seul protocole pour détecter les pathogènes ADN et ARN est indispensable pour une facilité d'application.

Une deuxième ligne de perspectives Une autre perspective est l'utilisation de mNGS en complément de la culture cellulaire, sur un large panel de LCR prélevés lors de méningites d'étiologie inconnue pour identifier tout éventuelle infection peu ou pas diagnostiquée en routine. Vingt prélèvements de LCR issus de patients présentant une méningite non documentée collectés pendant les étés 2018 et 2019 durant les pics épidémiques entre juin et aout, ont été inoculés selon un protocole que nous avons appelé « JNSPV » (« Je ne sais pas Virus, Prof. B. LA SCOLA) en partant sur cinq repiquages aléatoires pour huit semaines en culture cellulaire au laboratoire NSB3 en utilisant des cellules VeroE6 et MRC5. Des effets cytopathiques ont été mesurés chaque semaine afin d'identifier tout éventuelle culture microbienne. A l'issu de cinq repiquages, aucun effet cytopathique n'a

été observé et aucune culture positive n'a été détectée après cette durée d'incubation. Cela peut être dû à la non-conservation des échantillons LCR durant cette période ce qui a influencé la viabilité des pathogènes. Comme nous ne savons pas la nature de l'éventuel pathogène, nous avons protocolé tous les surnageants de culture en séquençage métagénomique en utilisant la technologie Oxford Nanopore MinION dans un premier temps après extraction à la fois de ADN/ARN suivant un protocole maison. Dans l'attente de résultat de la métagénomique nous allons continuer à repiquer chaque semaine à l'aveugle et de passer les surnageants en MinION suivant le même protocole. Comme seconde alternative, la métagénomique totale a été envisagée directement sur les LCR négatifs en culture après enrichissement du génome pathogène et déplétion du génome humain en suivant le protocole ADN/ARN pour identifier des pièces de génome pathogène potentiellement causatif de cette maladie.

Nos travaux de Thèse donnent une nouvelle perspective pour l'implantation d'un diagnostic rapide par métagénomique en temps réel des méningites communautaires à urgence vitale dans un délai comparable à celui des approches RT-PCR multiplexées utilisées en routine qui ciblent un nombre limité de pathogènes, sujettes à contamination moléculaire entrainant des faux positifs dans 2,63-25% des cas positives (29,72). Nous proposons au terme de nos travaux, de Thèse, que la détection de séquences génomiques des pathogènes dans le LCR sans PCR préalable peut être mise en place pour diagnostiquer les méningites ; y compris les méningites communautaires non documentées ; ainsi que les méningites liées aux soins (qui n'entraient pas dans le champ de nos travaux de Thèse). La constitution de bases de données locales adaptées et évolutives, est un point clé de cette mise en place. Cette approche piste pourrait mener au remplacement progressif des RT-PCR multiplex de routine par un seul protocole de métagénomique en temps réel, en rajoutant des informations supplémentaires sur le génotypage le profil d'antibiorésistance même en cas d'échec en culture, qui très compétitif en temps et en coût avec les systèmes multiplex actuels.

Dans cette perspective de mise en place en routine, nous sommes en train de remplacer progressivement les PCR multiplex par la technique RTM au laboratoire POC de l'IHU. Nous avons repris les données microbiologiques rétrospectives 2019-2021, en comptant le nombre de méningites communautaires diagnostiquées, en fonction du nombre de leucocytes/mL (**Figure 1**). Que nous prenions en compte les méningites documentées à Enterovirus ou non, nous avons observé un taux de documentation >16% à partir de ≥ 100 éléments/mL. Sur la base de cette observation, nous proposons une nouvelle stratégie diagnostique des méningites communautaires au laboratoire POC, comportant une première étape de détection moléculaire rapide du génome des Entérovirus par un procédé approprié (actuellement au POC IHU, le panel BioFire FilmArray®). Si le test BioFire FilmArray® est rendu positif, le chemin routine va être suivi pour le génotypage et l'inoculation en culture pour caractériser les co-infections bactériennes ainsi que le profil d'antibiorésistance du microbe. Dans le cas où le BioFire FilmArray® est négatif, les prélèvements de LCR présentant > 100 éléments/mL seront analysés par métagénomique ADN/ARN en utilisant le MinION et le iSeq, tel que développé au cours de nos travaux de Thèse, pour détecter toute infection non documentée en routine.

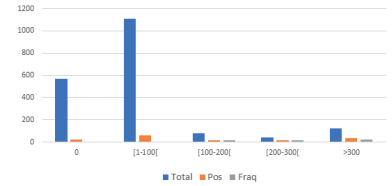
Dans une autre perspective si la RTM et la culture cellulaire suivie de métagénomique ADN/ARN est négative une investigation bio-informatique profonde

devrait analyser toutes les séquences non classifiées ou séquences dites « ADN poubelle » pour identifier des liens communs entre tous les cas négatifs afin de caractériser des séquences de pathogènes nouveaux ; ou bien des séquences insérées dans le génome humain par suite d'une infection.

Cela aussi nécessite la mise à jour régulière des bases de données génomiques des pathogènes, basée sur les données bibliographiques pour détecter toute nouvelle infection.

Figure 1

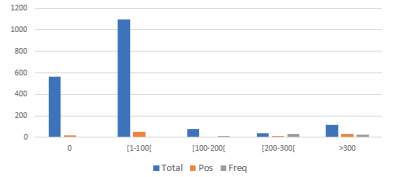
a)



Total positive (2019-2021) with Enterovirus infection

WBC	Total	Pos	%
0	569	20	2,64275554
[1-100[	1110	63	6,99095254
[100-200[	81	14	**16,1804993**
[200-300[	43	14	**16,1846154**
>300	124	33	**22,7899878**

b)



Total positive (201962021) without Enterovirus infection

WBC	Total	Pos	%
0	566	17	3,00353357
[1-100[	1094	52	4,75319927
[100-200[	77	8	**10,3896104**
[200-300[	39	12	**30,7692308**
>300	118	31	**26,2711864**

Figure 1 : Variation de nombre et fréquence des LCR positifs en fonction de nombre de leucocyte par ml. **a**) La fréquence des cas positifs totaux ne dépasse pas 7% si le nombre de leucocytes est inferieur à 100 éléments/ml, cependant cette fréquence peut dépasser les 22% en cas de leucocytes >100 élément/ml. **b**) Le pourcentage de cas documentés est < 5% en excluant les tests positifs à Entérovirus si le nombre de leucocyte est < 100. Dans la situation de pourcentage de cas positifs peut dépasser 30% si le nombre de leucocytes est > 100.

Abbreviation: WBC: white blood cell.

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ANNEXES

A. Evaluation d'une nouvelle stratégie de diagnostic et génotypage des SARS-COV-2

Préambule

A la fin de l'année 2020 Oxford Nanopore Technologies a développé un instrument de laboratoire dédié au diagnostic de l'infection de SARS-COV-2 appelé LamPORE. Sur la base d'une amplification isotherme Loop-mediated isothermal amplification (LAMP), le LamPORE peut diagnostiquer plus de 5.000 prélèvements par jour par amplification et séquençage direct en temps réel des produits de LAMP, pour la recherche de SARS-CoV-2. Nous en avons optimisé les paramètres d'utilisation. Pour une bonne interprétation des résultats du LamPORE, un nouvel algorithme a été élaboré afin d'éliminer tous les faux positifs générés par cet instrument. En plus du diagnostic de SARS-COV-2, nous avons exploité les données de séquençage pour tracer le variant Marseille 4 très fréquent dans notre région sur la base de deux mutations couvertes par les produits LAMP.

Article 9

LamPORE SARS-CoV-2 diagnosis and genotyping: A preliminary report.

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Letter to the editor

LamPORE SARS-CoV-2 diagnosis and genotyping: A preliminary report

Dear Editor,

The direct diagnosis of COVID-19 is mainly based on the reverse-transcription PCR (RT-PCR) detection of the SARS-CoV-2 RNA [1,2]. Recently, the LamPORE process has been developed for detecting the viral genome using reverse-transcription loop-mediated amplification (RT-LAMP) and sequencing the RT-LAMP product, following the LamPORE protocol on an Oxford Nanopore GridION instrument (Oxford Nanopore, Oxford, UK) [3]. Here, 264 nasopharyngeal swabs routinely submitted to BGI's Real-Time Fluorescent RT-PCR kit (BGI, Wuhan, China) (61 positive and 203 negative samples) at the IHU Méditerranée Infection, Marseille, France, were investigated in parallel using the LamPORE procedure. Briefly, the LamPORE procedure combines an isothermal amplification step for 30 min at 65 °C, performed in a 50-µL reaction containing 20 µL RNA previously extracted on a Kingfisher instrument, using the MagMax™Viral/Pathogen Kit (ThermoFisher, St. Austin, USA), 5 µL primer mix and 25 µL of LamPORE master mix, targeting three SARS-CoV-2 genes: ORF1a, the envelope (E) and nucleocapsid (N) genes in addition to the human actin mRNA as an internal control. In addition, 2 µL of each amplified sample was incorporated into the LamPORE library preparation, as previously described [3] and the library was sequenced on a GridION instrument for one hour. LamPORE assays incorporated water as negative control into the process, as provided for by the manufacturer. The results of the LamPORE analysis were interpreted as positive or negative and were automatically generated in a pdf file as follows: the test was considered positive when the sum of reads generated by the three targets ≥ 50, and negative when the number of reads < 20. A test was considered to be inconclusive when the number of reads was between 20 and 50. To eliminate any source of contamination, the positive and negative samples were separated by two empty columns on the plate within the library preparation. Using the strict interpretation algorithm proposed by the manufacturer, all 61 nasopharyngeal swabs which were routinely positive for SARS-CoV-2 were found to be positive by LamPORE (100 % sensitivity) but 23/203 of RT-PCR negative nasopharyngeal swabs were identified as being positive by LamPORE (88.7 % specificity). These 23 nasopharyngeal swabs exhibited RT-PCR Ct values > 40, reflecting a lack of SARS-CoV-2 viability [4,5]. Referring to the RT-PCR results, the LamPORE reaction was positive for the three targets in all the RT-PCR-positive samples, contrary to the 23 discordant swabs.

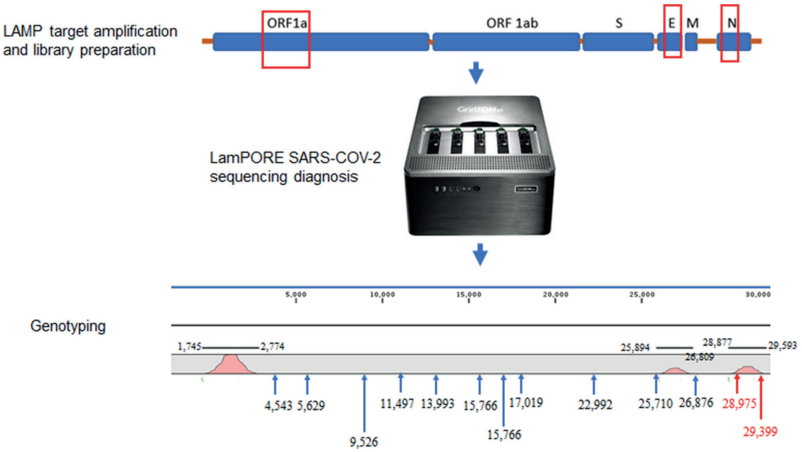

Fig. 1. LamPORE diagnosis strategy for SARS-COV-2 detection and genotyping. LAMP amplification and library preparation were followed by library sequencing on the LamPORE instrument. A genotyping step was added to specifically detect SARS-COV-2 genotype 4.

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Analyzing the 23 discordant results, we observed that for a result to be consistent with RT-PCR, all target genes should have at least one read mapped on them and the sum of the three targets should also read ≥50. Applying this new interpretation rule allowed us to achieve 100 % sensitivity and 100 % specificity, in the 61-sample collection we investigated. This new interpretation rule consisted in eliminating false positives by applying the "=IF(AND((ORFa1+N + E≥50); (ORFa1*N*E>0));"pos";"neg")" formula directly in the Excel file. In a second step, we used sequences to detect the SARS-CoV-2 strain Marseille genotype 4 also referred as 20AEU2, the most prevalent genotype in the Marseille area in the period under consideration. LamPORE theoretically generates substitutions G28,975 T and G29,399A in the nucleocapsid gene, among the 13 mutations specific for genotype 4 (20AEU2) (Fig. 1). Here, LamPORE detected two Marseille genotype 4, based on two sequences exhibiting only the G28,975 T mutation and one sequence exhibiting the mutation G29,399A.

Although preliminary, the data here reported confirm that LamPORE is an appropriate method for the rapid direct detection of SARS-CoV-2 RNA in nasopharyngeal swabs, with a capacity of 480 tests per hour, depending on the adoption of the interpretation rule we report here. This method is also promising for one-shot genotyping of SARS-CoV-2 depending on further experimental improvements, and may offer a new, alternative way for detecting SARS-CoV-2 and conducting genomic surveillance.

Authors' contribution

MM contributed to the experimental design, performance of the work, data analysis, interpretation and writing. AH collected samples, carried out data analysis and writing. BL organized the work. DJ conducted bioinformatic data analysis. BM contributed to the experimental design. PEF and DM contributed to critically reviewing the manuscript, interpreting the data, coordinating and directing the work. All authors declare that they have read and approved the manuscript.

Ethics

Only nasopharyngeal residual fluid left from standard-of-care clinical laboratory testing was used. All specimens had been referred to our laboratory for diagnostic purposes between 1 September and 1 November 2020. The study was approved by our Institute's Ethics Committee under number 2020−016603.

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Declaration of Competing Interest

Reagents for the LamPORE instrument were provided by Oxford Nanopore Technology, Oxford, UK. However, the supplier did not interfere in the experimental plan, data interpretation, manuscript preparation or submission.

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B. Nouvelles méthodes de diagnostic et de surveillance génomique de la tuberculose.

Préambule

Le diagnostic moléculaire de la tuberculose est basé sur le GeneXpert en investiguant par des PCR nichées la présence des espèces de *Mycobacterium tuberculosis* complexes, aussi bien la résistance à la rifampicine. Des étapes supplémentaires *in-vitro* sont indispensables pour caractériser la mycobactérie et son profil de résistance à l'antibiotique. Afin d'aller plus vite dans l'identification avec plus de précision l'espèce causative de la tuberculose ainsi que ses variations possibles des espèces et des lignages nous avons combiné deux protocoles de culture cellulaire Shell-Vial et le séquençage en temps réel pour identifier à la fois la présence de l'espèce causative de la tuberculose, son lignage et son profil de résistance aux antibiotiques dès les vingt premières minutes de séquençage. Cette détection est consolidée par le séquençage de génome entier directement sur une culture solide pour maximiser la précision d'identification de l'espèce, lignage et son profil d'antibiorésistance afin de proposer un traitement adéquat aux patients.

Dans un autre temps, la procédure de séquençage en temps réel a été adoptée et implémentée comme une alternative pour aller plus vite dans le diagnostic de la Tuberculose ganglionnaire ainsi que le génotypage, l'antibiogramme *in-silico* directement à partir de cultures et de prélèvements cliniques en cas d'échec de la culture.

Article 10

Real-time next-generation sequencing on shell-vial culture to contribute to diagnosis of lymphatic tuberculosis: a case report

Madjid Morsli [1], Marc Faltot [2], Hélène Astier [3], Erwan Le Dault [3], Bernard Chaudier [3], Eric Garnotel [3], Sophie Alexandra Baron [1], Michel Drancourt [4]

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Case Report

Real-time next-generation sequencing on shell-vial culture to contribute to diagnosis of lymphatic tuberculosis: a case report

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ABSTRACT

Lymph node tuberculosis is a of limited clinical suspicion form of *Mycobacterium tuberculosis* infection. After 15 days incubation in a cellular culture and directly from the supernatant, 11 minutes of Oxford Nanopore MinION sequencing provided a preliminary result of an antibiotic-susceptible *M. tuberculosis* Indo-Oceanic lineage strain. Oxford Nanopore MinION sequencing is a promising tool for optimising the laboratory diagnosis of lymph node tuberculosis.

1. Introduction

Mycobacterium tuberculosis (*M. tuberculosis*) complex mycobacteria are responsible for tuberculosis, which remains a major cause of morbidity and mortality in human populations worldwide (World Health Organization 2019). The annual incidence is over 10 million cases of infection and up to 1.4 million deaths, as reported by the World Health Organization in 2019 (World Health Organization 2019). While pulmonary tuberculosis is the main clinically recognised form of the infection, lymph node tuberculosis is of limited clinical suspicion form, in alignment with the idea that tuberculosis pathogens affect the lungs tissue and subsequently the reticuloendothelial system, causing an infection of the lymphatic system (Pai *et al.*, 2016).

The diagnosis of lymph node tuberculosis is challenging for clinical microbiology. Besides the fact that lymph node tuberculosis may be clinically ignored for long periods, notably in the case of profound lymph node disease, and that sampling of such seriously diseased lymph nodes may be hazardous, microbiological documentation remains unsatisfactory (Pai *et al.*, 2016; Fellag *et al.*, 2019). Routinely used GeneXpert MTB/RIF® assays (Cepheid, Sunnyvale, CA) provide rapid but only limited information as they do not distinguish between dead and live mycobacteria, nor do they distinguish between species within the *M. tuberculosis* complex. Although *M. tuberculosis stricto sensu* is the main causative agent (Ghariani *et al.*, 2015), *Mycobacterium bovis* (Ghariani *et al.*, 2015), *M. bovis* BCG (Fellag *et al.*, 2020), *Mycobacterium canettii* (Blouin *et al.*, 2014), *Mycobacterium africanum* (Geerdes-Fenge *et al.*, 2018), *Mycobacterium caprae* (Cvetnic *et al.*, 2007) and *Mycobacterium orygis* (Marcos *et al.*, 2017) are also rarely reported in this situation (Zhou *et al.*, 2019; Fellag *et al.*, 2019). Moreover, sensitivity of the Xpert MTB/RIF or Xpert Ultra tests on lymph node samples remains moderate, between 70 and 81.6% for a specificity of 100% (Kohli *et al.*, 2021).

Conventional culture on egg-based medium, the gold standard for isolating *M. tuberculosis*, failed in lymph nodes diagnosis in 70% of cases (Benjelloun *et al.*, 2015).

Here, we combined the shell-vial protocol (Fellag *et al.*, 2019) with Next-Generation Sequencing (NGS) (Zhou *et al.*, 2019; Votintseva *et al.*, 2017) in order to optimise the laboratory diagnosis of lymph node tuberculosis.

2. Case presentation and methods

A 22-year-old man originating from India, who had been living in France for 12 years, was admitted in our unit for a persistent fever, chills and cervical lymphadenopathy. The patient weighed 73 kg and reported no weight loss. A physical examination found no further organomegalies. A medical interview reported a family history of tuberculosis diagnosed in 2011 in his mother and his uncle. A

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computerized tomography scan showed necrotic lymph nodes extending from the left retropharyngeal region to the left supra-ventricular region, infracentimetric non-necrotic lymph nodes in periportal and sub-diaphragmatic regions. The pathological examination of the left cervical lymph node biopsies showed giganto-cellular epithelioid granuloma with caseous necrosis. A GeneXpert MTB/RIF® ultra-assay identified a rifampin-susceptible *M. tuberculosis* complex. The lymph node sample was inoculated on both MB/BACT MB liquid medium (Biomérieux, Marcy L'Etoile, France) and on solid medium, Coletsos (Asmar and Drancourt, 2015) and Lowenstein-Jensen. The liquid medium was positive after 45 days, and replicate on solid medium grew after 48 days. Solid medium cultures were positive after 70 days of incubation. In parallel, 200 μL of biopsy fragments were inoculated onto MRC5 cells (human foetal pulmonary cells, RD-Biotech, Besançon, France). Briefly, after 1-hour incubation, the tube is centrifuged, the supernatant collected and 1.5 mL of M10 is added to the jewel tube that is then incubated at 37°C under 5% CO2 for 8 weeks (Fig. 1). Cells culture is checked every 3 days and a Zielh-Neelsen staining is performed. The culture medium is changed once a week. After 15 days post-inoculation, acid-fast bacilli were observed on shell vial cultures, while the negative control remained sterile (Fig. 1). Total DNA extracted from 500 μL of the shell-vial supernatant as previously described (Fellag *et al.*, 2019) and 47 μL (1 ng/μL) DNA were incorporated into an Oxford Nanopore library preparation as previously described (14), then sequenced for 2 hours on a MinION instrument (Oxford Nanopore, Oxford, UK).

3. Results and discussion

A first 11-minute MinION sequencing run generated 92,000 reads, including 229 *M. tuberculosis* complex reads and 224 *M. tuberculosis sensu stricto* reads (Fig. 1) analysed online using Oxford Nanopore tools designed for real-time sequencing and the EP2ME software (version 2019.11.11-2920621) (Lu *et al.*, 2016). After two hours of sequencing, the blast of generated reads after assembling on a Canu assembler available online on Galaxy Europe (Version 1.8) yielded a *M. tuberculosis* strain FDAARGOS-756 (GenBank accession no.: CP054014). Total reads were extracted and mapped to the *M. tuberculosis* strain FDAARGOS-756 complete genome using CLC Genomics Workbench software version 7.5.0 (Qiagen). Of the 744,732 reads in total, 2,811 were mapped to the reference genome and 4,143,715 nucleotides were matched to the reference sequence, resulting in a 93.8% genome coverage with the reference genome (Fig. 1). Direct analysis of the generated reads using the TB-Profiler (Version 2.8.4 +galaxy1) identified an Indo-Oceanic lineage (L.1.1.3) which was susceptible to all antibiotics. In parallel, this genome was sequenced with iSeq technology (Illumina, San Diego, CA) using the Illumina Nextera XT paired-end protocol (Smith *et al.*, 2020). Results were consistent with those obtained using MinION sequencing (Appendix 1) (Smith *et al.*, 2020; Chan *et al.*, 2020).

Using a shell-vial culture protocol, we reduced the delay before a positive culture of 55 to 78 days compared to standard culture. The Oxford nanopore technology genome sequencing identified the strain at the species level in 11 min and give a complete genotype in 2 hour while it takes 17 hour to sequence the complete genome using Illumina technology. The "real-time" function of the Oxford nanopore technology led to an interesting time saving, the first reads can be analysed without stopping the sequencing process. The latest published works (Votintseva *et al.*, 2017; Smith *et al.*, 2020), as well as our own experience, confirm Oxford technology is competitive in terms of cost and handling time. This cost becomes competitive compared to other molecular biology methods that require several PCRs to obtain the same level of information. The obtained reads with both genomic strategies led to the same identification and antibiotic susceptibility even though reads number and coverage were lower with MinION. Integrating both MinION and the shell-vial protocol will help refine and expand the diagnosis of lymph-node tuberculosis primarily established by the GeneXpert MTB/RIF® ultra-assay (Ghariani *et al.*, 2015). This "open" approach would even make it possible to screen for any intracellularly cultured microorganisms, specifically for the search of aetiological agents of lymphadenopathy.

4. Ethics

All data were generated as part of routine work at the Assistance Publique-Hôpitaux de Marseille (Marseille University hospitals), and this study is the result of routine clinical management. No specific clinical sampling was performed in the course of this study.

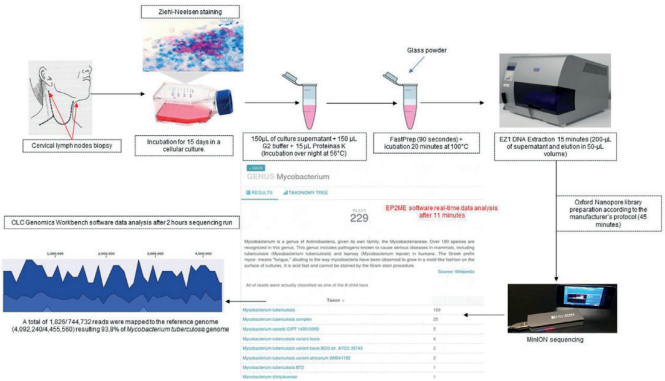

Fig. 1. *Two-week investigation of lymph node tuberculosis combining shell-vial and whole genome sequencing.* A shell-vial culture inoculated with a lymph node biopsy was detected positive by Ziehl-Neelsen staining after 2 wk of incubation. The shell-vial supernatant (500 μL) was inactivated, centrifuged for 15 min at 13,000 g and the pellet was incubated in 150 μL of G2 buffer and 15 μL of proteinase K overnight at 56°C. After treatment with glass powder and incubation at 100°C for 10 min, DNA was extracted from 200 μL of supernatant using an EZ1 DNA kit (Qiagen) and eluted in 50 μL. A 47-μL volume of DNA was used for NGS library preparation according to the Oxford Nanopore protocol. Real-time data analysis was performed using EP2ME software and the *M. tuberculosis* genome was recovered by CLC software.

Author contributions

MM performed the experimental design, data analysis, interpretation, and writing. MF, HA and EG collected samples and performed routine analyses. ELD and BC collected clinical data and samples. SAB performed data analysis, validation, and writing. MD contributed to critically reviewing the manuscript, data interpretation and validation. All authors declare that they have read and approved the manuscript.

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Declaration of competing interest

The authors have no conflicts of interest to declare.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.diagmicrobio.2021.115492.

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Résumé

Les méningites communautaires sont des urgences vitales, dont le pronostic est partiellement associé à l'agent microbien pathogène et son génotype. Plus de 100 micro- organismes différents ont été impliqués dans les infections de système nerveux central après leur détection et leur identification dans le liquide céphalo-rachidien (LCR), à travers le monde. La culture de LCR a longtemps été le gold standard du diagnostic des infections de système nerveux central mais actuellement, ce diagnostic de routine microbiologique est basé sur la détection par PCR multiplexe en temps réel des pathogènes les plus fréquents. Cependant, le génotypage des microbes responsables tel que les Entérovirus qui couvrent plus de 300 sérotypes différents, dont 110 infectent les patients, ainsi que le génotypage et l'antibiogramme des bactéries pathogènes, nécessitent des investigations *in-vitro* supplémentaires. Notre étude rétrospective de 20,779 LCR prélevés dans le cadre du diagnostic des méningites communautaires au cours de 61 mois, analysés dans les laboratoires de microbiologie clinique de Nîmes et Marseille a montré l'absence de documentation dans plus de 89% des cas. La métagénomique NGS est un outil potentiel pour le diagnostic direct des méningites infectieuses à partir de LCR en détectant le génome pathogène sans PCR préalable. Dans ce travail de Thèse, nous avons répondu à quatre problématiques : 1) Mise à jour du répertoire des agents pathogènes causatifs de méningites, détectés par métagénomique NGS directe du LCR. 2) Epidémiologie des méningites communautaires à Nîmes et Marseille. 3) Amélioration de diagnostic et génotypage des méningites à Entérovirus. 4) Développement et implantation d'un protocole "one-shot" utilisant la métagénomique en temps réel pour le diagnostic, le génotypage et l'antibiogramme *in-silico* des méningites au laboratoire point-de-soins (POC).

Mots-clés : Méningites communautaires, liquide céphalorachidien, infection, épidémie, méningites non-documentées, étiologie, génome pathogène, métagénomique, résistance, génotypage, séquençage en temps réel, NGS, diagnostic, point-de soins.

Abstract

Community-acquired meningitis is a life-threatening condition, whose prognosis is partially dependent on the causative pathogen and its genotype. More than 100 different microorganisms have been involved in central nervous system infections after their detection and identification in cerebrospinal fluid (CSF), worldwide. The CSF culture was for a long time the gold standard for the diagnosis of central nervous system infections but nowadays, this routine microbiological diagnosis is based on the detection by multiplexed RT-PCR of the most frequent pathogens. However, the genotyping of causative pathogens such as Enteroviruses which cover more than 300 different serotypes, of which 110 infect humans, as well as the genotyping and antibiogram of pathogenic bacteria, require additional *in-vitro* investigations. Our retrospective study of 20,779 CSFs collected for the diagnosis of community-acquired meningitis during 61 months, analyzed in the clinical microbiology laboratories of Nîmes and Marseille showed the absence of documentation in more than 89% of cases. Metagenomics NGS is a potential tool for the direct diagnosis of infectious meningitis from CSF samples by detecting the pathogenic genome without prior PCR. In this thesis work, we have addressed four issues: 1) Update of the repertoire of meningitis causative pathogens detected by direct metagenomic NGS of CSF. 2) Epidemiology of community-acquired meningitis in Nîmes and Marseille. 3) Improvement of the diagnostic and genotyping of Enterovirus meningitis. 4) Development and implementation of a "one-shot" protocol using real-time metagenomics for diagnosis, genotyping, and *in-silico* antibiotic susceptibility testing of community-acquired meningitis in the point-of-care (POC) laboratory.

Keywords: Community-acquired meningitis, cerebrospinal fluid, infection, outbreak, undocumented meningitis, etiology, pathogen genome, metagenomics, resistance, genotyping, real-time sequencing, NGS, diagnosis, point-of-care.