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Détection des bactéries entéropathogènes: approche polyphasique

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COMPOSITION DU JURY

Mr le Professeur Jean-Louis Mège	Président du jury
Mme le Professeur Marie-Cécile Ploy	Rapporteur
Mr le Professeur Bertrand Picard	Rapporteur
Mr le Professeur Michel Drancourt	Directeur de thèse

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"Une théorie est scientifique si et seulement si elle susceptible d'être réfutée ;

elle n'est pas vraie, mais tout au plus admise provisoirement."

Karl Popper

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. Par ailleurs, 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.

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Sommaire

Résumé

Le corps humain est un ensemble de microflores où cohabitent bactéries, archées, virus et micro-eucaryotes. Ces écosystèmes complexes sont appelés microbiotes. Parmi ceux-ci figure le microbiote intestinal qui compte 10^{11} à 10^{14} bactéries/g de selle. Les modifications de la flore intestinale peuvent être à l'origine de pathologies comme les diarrhées infectieuses. Il s'agit d'un véritable problème de santé publique puisqu'environ 2,16 millions de décès sont liés à cette pathologie chaque année. Les virus intestinaux jouent un rôle prépondérant mais les infections bactériennes restent également importantes. Le diagnostic de ces infections bactériennes demeure compliqué puisque le microbiote intestinal comporte 75% d'espèces non cultivables. De plus, on ne dispose pas réellement d'une liste exhaustive des bactéries pouvant être responsables de diarrhées infectieuses. Nous avons donc choisi d'étudier le microbiote intestinal dans des selles normales et pathologiques, par une approche polyphasique alliant une étape préliminaire de concentration des selles diarrhéiques par la lyophilisation, à des techniques de culture et des méthodes de biologie moléculaire. Pour cela nous avons mis au point une nouvelle technologie pour la détection des entéropathogènes par hybridation sur puce à ADN permettant la détection des bactéries et des virus ADN entéropathogènes, en présence d'un témoin archae. Notre outil permet le diagnostic multiplexe des diarrhées infectieuses puisque nous avons correctement identifié un adénovirus et la bactérie *Campylobacter jejuni* présents dans une même selle. Il s'agit de la première puce ADN multiplexée permettant la détection des bactéries et des virus (ADN) entéropathogènes. Une modification du protocole d'extraction des acides nucléiques est envisagée afin de permettre la détection des virus à ARN tels que les rotavirus ou les calicivirus qui sont actuellement prépondérants. En parallèle, nous avons mis au point un protocole de dilution permettant l'identification des bactéries présentes à une concentration

$\geq 10^{10}$ unités formant colonies (UFC)/g de selles. Ce protocole consiste à diluer des selles dans du tampon phosphate stérile à 10^{-10} et à ensemencer cette dilution sur un milieu non sélectif. On identifie ensuite par spectrométrie de masse MALDI-TOF toutes les colonies obtenues après culture à 37°C pendant 24 heures. Cette étude a été menée sur un total de 347 selles ayant une consistance normale et 317 selles diarrhéiques. Nous n'avons pas observé de différence significative au niveau de la composition de ces deux groupes avec 42 espèces bactériennes retrouvées dans le groupe des selles non-diarrhéiques et 45 espèces bactériennes pour le groupe des selles diarrhéiques. Nous avons observé une corrélation négative entre la présence d'un rotavirus ou d'un adénovirus et certaines espèces bactériennes résidentes du microbiote intestinal.

Au total, ce travail de thèse a montré la difficulté des méthodes de détection rapide des bactéries entéropathogènes à partir des selles, difficulté à laquelle la mise au point de la puce à ADN au sein de notre laboratoire permet de répondre. Parallèlement, nous avons montré de façon inattendue, les relations complexes entre certains virus entéropathogènes et la flore bactérienne aérobie résidante du microbiote intestinal. Notre puce ADN est d'autant plus utile pour l'étude de ces interactions, puisqu'elle permet pour la première fois une détection multiplexée des bactéries et des virus ADN entéropathogènes.

Mots-clés: microbiote, intestinal, polyphasique, puce à ADN, diarrhée, bactérie, virus, archée, lyophilisation, culture.

Abstract

The human body is a collection of microflora where cohabit bacteria, archaea, viruses and micro-eukaryotes. These complex ecosystems are called microbiota. Among these is the intestinal microbiota that counts 10^{11} to 10^{14} bacteria/g of stool. Changes in the intestinal flora can cause diseases such as infectious diarrhea. This is a real public health problem since about 2.16 million deaths are related to this disease each year. Enteric viruses play a preponderant role but bacterial infections are also important. The diagnosis of bacterial infections is complicated because the intestinal microbiota includes 75% non-cultivable species. In addition, there is not really a list of bacteria could be responsible for infectious diarrhea. We therefore decided to study the intestinal microbiota in normal stool and also pathological stools by a polyphasic approach combining a preliminary step of diarrheal stools concentration by lyophilization, with cultivation techniques and molecular biology methods. We developed a new technology for the detection of enteropathogens by hybridization on DNA microarray for the detection of bacteria and enteric viruses (DNA) in the presence of a control archaea. Our tool allows multiplexed diagnostic of infectious diarrhea since we correctly identified an adenovirus and the bacteria *Campylobacter jejuni* present in a same sample. This is the first DNA microarray for multiplex detection of enteropathogenic bacteria and viruses (DNA) enteropathogens. An improvement of our protocol for nucleic acid extraction is proposed to allow the detection of RNA viruses such as rotavirus and calicivirus which are currently dominant. In parallel, we developed a dilution protocol for the identification of bacteria at concentrations $\geq 10^{10}$ colony forming units (CFU)/g of feces. This protocol consists of diluting stool specimens in sterile phosphate buffer at 10^{-10} and seeding this dilution on non-selective media. Then, we identified by MALDI-TOF all colonies obtained after culturing at 37 ° C for 24 hours. This study was conducted on a total of 347

non-diarrheal stool specimens and 317 diarrheal specimens. We did not observed any significant difference in the composition of these two groups with 42 bacterial species found in the group of normal stools and 45 bacterial species for the group of diarrheal stools. We observed a negative correlation between the presence of rotavirus or adenovirus and resident bacterial species of the intestinal microbiota.

Finally, this work has shown the difficulty of methods for rapid detection of enteric pathogens from stool, difficulty in which the development of DNA microarray in our laboratory can respond. In parallel, we showed unexpectedly, complex relationships between some enteric viruses and resident aerobic bacterial flora of the intestinal microbiota. Our DNA microarray is particularly useful for the study of these interactions, since it allows for the first time, the multiplexed detection of bacterial and viral DNA enteropathogens.

Keywords: microbiota, intestinal, polyphasic, DNA microarray, diarrhea, bacteria, virus, archaea, lyophilization, culture methods.

Introduction

Le corps humain comporte des bactéries, des virus, des archées, des levures et des champignons filamenteux interagissant entre eux [1**]** au sein de communautés appelées microbiotes. Parmi ceux ci figure le microbiote intestinal composé de 10^{11} à 10^{14} bactéries selon l'âge [2]. A la naissance, le tube digestif d'un nourrisson est stérile, la colonisation se fait dès la rupture des membranes fœtales et se poursuit pendant les premiers mois de vie [3]. La composition de la flore intestinale des nourrissons va dépendre du type d'alimentation: l'allaitement par le lait maternel favorise le développement d'une flore majoritairement composé de *Bifidobacterium*, alors qu'une alimentation à base de lait de vache favorise le développement d'une flore composée de bactéries du genre *Clostridium* [4,5].

Après 48 heures de vie, la flore fécale d'un nourrisson est composée de 10^4 à 10^6 unités formant colonies (UFC)/g de selle [6]. La phase de diversification de la flore intervient au moment où l'enfant commence à recevoir une alimentation plus variée. On voit alors se développer une flore composée d'entérobactéries et de streptocoques. A la fin de la première année de vie, la flore intestinale présente une composition semblable à celle d'un adulte. Le nombre de bactéries anaérobies strictes et alors beaucoup plus important. Le microbiote intestinal d'un adulte est composé de bactéries appartenant à huit des 55 divisions bactériennes [7]. Les phylums dominants sont les *Firmicutes* (14-31%), les *Bacteroidetes* (9-42%) et les *Actinobacteria* (7-10%) [8]. 80% de ces espèces bactériennes dominantes sont spécifiques à un individu donné [9,10]. Plusieurs travaux rapportent un lien significatif entre composition de la flore intestinale et pathologies intestinales: maladie de Crohn [11], syndrome du colon irritable [12], colite ulcérante [13], ou métaboliques: obésité [14,15], diabète de type 1 [16], maladie cœliaque ou intolérance au glucose [17].

Une autre maladie liée à la modification de la flore bactérienne est l'entérite aigue qui se manifeste essentiellement par une diarrhée infectieuse. Il s'agit d'un problème de santé publique puisque l'OMS classe les diarrhées infectieuses parmi les cinq maladies les plus meurtrières dans le monde (www.who.int/fr/) derrière les maladies respiratoires, ou l'infection par le VIH. Chaque année, environ 2.16 millions de personnes décèdent des suites d'une diarrhée infectieuse et 70% de ces décès surviennent chez des enfants. Les diarrhées infectieuses se caractérisent par une émission quotidienne de selles trop abondantes, liquides ou très molles (poids supérieur à 300 g/j). Les autres symptômes peuvent être de fortes douleurs abdominales accompagnées de vomissements et parfois de fièvre, et présence de sang dans les selles. La mortalité associée à la maladie est due à la déshydratation importante qui survient très rapidement après l'émission répétée de selles liquides. Le principal traitement des diarrhées infectieuses consiste en une réhydratation rapide du patient. 33 espèces bactériennes sont connues pour être responsables de diarrhées infectieuses telles que *Salmonella* spp., les différents pathovars d'*Escherichia coli, Shigella* spp., *Yersinia* spp., *Campylobacter* spp., ainsi que *Clostridium difficile* [18]. Les virus intestinaux jouent également un rôle prépondérant puisqu'ils sont responsables de près de 50% des cas de diarrhées infectieuses [19]. Parmi ces virus, on compte les rotavirus, les torovirus, les coronavirus, les astrovirus, les entérovirus, les adénovirus mais également les norovirus qui sont aujourd'hui la principale cause d'épidémies d'entérites dans le monde [20,21]. Dans les laboratoires hospitaliers, le diagnostic des diarrhées infectieuses d'origine bactérienne repose essentiellement sur la culture des pathogènes. Cependant 75% des espèces bactériennes composant le tractus gastro-intestinal ne sont pas des espèces cultivables [22,23] et l'identification d'un pathogène peut prendre de 48 à 72 heures. Ce sont deux freins à la prise en charge d'un patient souffrant d'une diarrhée bactérienne.

Les premiers travaux d'exploration du microbiote intestinal étaient basés sur des techniques de culture [1,24-26]. Ces études ont permis de caractériser environ 400 espèces bactériennes cultivables. Dans le laboratoire de la Timone, Marseille, la «culturomic» a également permis d'identifier plus d'une dizaine de nouvelles espèces bactériennes jamais décrites dans le microbiote intestinal humain. Ces études ont fait l'objet d'articles scientifiques prochainement publiés. L'apparition des outils de biologie moléculaire [27-32] à permis de compléter les données de culture. Le plus souvent ces outils sont basés sur l'identification du gène universel 16S ARNr codant pour une sous-unité du ribosome [33]. Ces outils, tels que la «polymerase chain reaction» (PCR), la real-time PCR (RT-PCR) présentent de nombreux avantages à savoir une réduction considérable du temps de diagnostic, la possibilité d'identifier des espèces non cultivables et enfin la semi-automatisation des méthodes. Le coût des ces techniques ainsi que la possibilité de n'identifier qu'un seul pathogène par analyse sont les points négatifs des outils de biologie moléculaire. Les puces ADN, dont la première utilisation date de 1993 [34,35] présente l'avantage de pouvoir détecter plusieurs pathogènes présents dans un même échantillon au cours d'une seule analyse. La puce ADN consiste en un support solide, généralement une lame de verre, sur laquelle sont fixées des sondes oligonucléotidiques complémentaires de gènes universels ou de gènes de virulence des bactéries recherchées. Ces puces à ADN sont couramment utilisées pour l'exploration de flores complexes telles que le microbiote respiratoire, le microbiote oral, le microbiote vaginal mais également le microbiote intestinal [35].

Notre travail de thèse a porté sur l'exploration polyphasique du microbiote intestinal. Nous avons utilisé une puce à ADN mise au point dans notre laboratoire, mais également des techniques basées sur la culture des selles après utilisation d'un protocole de dilution pour

l’exploration de la flore bactérienne et virale dans des selles contrôles, c’est à dire ayant une consistance normale, et dans des selles diarrhéiques. Une technique spécifique de préparation des selles diarrhéiques a été mise au point.

Aperçu de la thèse

Au cours de notre thèse, nous avons dans un premier temps étudié les bactéries aérobies du tractus gastro-intestinal présentes à des concentrations supérieures à 10^{10} UFC/g de selle en partant de l'hypothèse que certaines bactéries entéropathogènes se multipliant dans le tube digestif, seraient présentes à fort inoculum dans les selles. Pour cela, nous avons collecté un total de 664 selles, dont 347 selles non-diarrhéiques et 317 selles diarrhéiques entre avril 2010 et avril 2011 au sein du laboratoire hospitalier de la Timone. Nous avons mis en place un protocole de dilution qui consistait à ensemencer une dilution 10^{-10} des selles sur une gélose au sang et à identifier les colonies par spectrométrie de masse MALDI-TOF, après 24 heures d'incubation à 37°C. Cette étude a permis de réfuter notre hypothèse de départ. En effet, la plupart des pathogènes retrouvés en routine au laboratoire de bactériologie de la Timone n'étaient pas mis en évidence par notre protocole de dilution. De plus, nous n'avons pas observé de différences significatives entre les espèces bactériennes cultivées à des concentrations supérieures à 10^{10} UFC/g de selle entre le groupe des selles moulées et les selles diarrhéiques. De façon inattendue, nous avons observé une corrélation négative entre la détection de virus entéropathogènes et certaines bactéries de la flore intestinale à des concentrations $\geq 10^{10}$ UFC/g de selle. En effet, la présence d'un rotavirus ou d'un adénovirus est corrélée à une diminution significative du nombre de bactéries aérobies présentes à des concentrations supérieures à 10^{10} UFC/g de selle. Nous avons mis au point un protocole permettant de tester une interaction directe entre les virus et les bactéries au sein du microbiote intestinal dans le cas d'une diarrhée infectieuse. Pour cela, nous avons mis en culture un rotavirus humain (fourni par le Professeur P. Pothier, Dijon, France) et nous en avons préparé une solution calibrée à 10^7 unité formant particule (UFP)/ml. D'autre part nous avons mis en culture différentes souches d'*Escherichia coli* obtenues durant notre étude et

nous en avons préparé une solution calibrée à 10^6 UFC/ml. Nous avons mélangé 500 µl de ces deux solutions que nous avons déposées sur une gélose au sang. Des observations ont été réalisées après 24, 48 et 72 heures d'incubation à 37°C. En parallèle, un témoin négatif a été réalisé en remplaçant la solution bactérienne par de l'eau stérile. Le but de ce protocole était de mettre en évidence les interactions entre bactéries et virus intestinaux en observant d'éventuelles plages de lyse sur la gélose. Au terme de ce travail, aucune plage de lyse n'a été observée dans chacun des deux groupes. Nous avons alors émis l'hypothèse que cette diminution de la flore intestinale dans le cas des diarrhées infectieuses était en partie due à la dilution des selles et également de la flore bactérienne, par la quantité d'eau anormalement présente dans les selles diarrhéiques. Nous avons alors mis au point un protocole de concentration des selles par lyophilisation suivi d'une extraction d'ADN semi automatisée dans le but de faciliter l'étude des selles diarrhéiques par des techniques de biologie moléculaire. L'extraction d'ADN comporte une première partie automatisée sur un automate EZ1 (Qiagen, Courtaboeuf, France) suivie par une étape d'extraction manuelle au phénol-chloroforme afin de purifier les ADN extraits. La dernière partie de cette thèse a consisté à mettre au point une puce ADN originale permettant d'améliorer le diagnostic des diarrhées infectieuses au sein du laboratoire de bactériologie de la Timone, dans lequel l'identification du pathogène responsable de diarrhée n'est réalisée que dans moins de 10% des cas. Nous avons choisi un outil permettant de contourner les problèmes d'identification liés à la culture et permettant également une détection multiplexe dans le cas des co-infections. Il s'agit de la première puce ADN permettant la détection multiplexée des bactéries et virus (ADN) entéropathogènes. D'autres puces ADN ont été mises en place, mais ne permettent la détection que d'une ou quelques bactéries entéropathogènes [36-38] ou de quelques virus intestinaux séparément [39]. La puce ADN que nous avons développée consiste en une lame

de verre sur laquelle sont fixées des sondes nucléotidiques de 60 paires de bases permettant l'identification de 33 bactéries et sept virus intestinaux responsables d'infections intestinales. Nous avons également choisi de fixer sur cette puce ADN une sonde permettant l'identification de *Methanobrevibacter smithii*. Cette archée nous sert de contrôle interne puisqu'elle est détectée dans les selles, chez 95,7% des individus [40].

Ce travail a permis de réfuter notre hypothèse de départ concernant la présence des pathogènes entériques en grande quantité dans le tractus gastro-intestinal. En effet, la plupart des pathogènes identifiés dans le laboratoire de bactériologie de la Timone n'ont pas été retrouvés à des concentrations $\geq 10^{10}$ UFC/g selle. Nous avons également pu constater que les puces à ADN facilitent l'analyse de plusieurs échantillons en même temps et également permettent une analyse multiplexe pour un même échantillon, mais restent une technologie nécessitant un investissement important. Le développement de nouvelles méthodes d'extraction des acides nucléiques totaux (ADN et ARN) et leur hybridation, permettra d'élargir le spectre de détection de cette première puce multiplexée.

CHAPITRE 1:

DNA microarrays for the diagnosis of infectious diseases

Revue

DNA microarrays for the diagnosis of infectious diseases

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Q1 General review

DNA microarrays for the diagnosis of infectious diseases

Puces à ADN pour le diagnostic des infections bactériennes

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Abstract

The diagnosis of bacterial infections relies on isolation of the bacterium, which is rarely achieved when needed for patient management. Furthermore, culture is poorly suited to the diagnosis of polymicrobial infections. Finally, a syndromic approach should target both bacteria and viruses causing the same syndrome. The detection of specific DNA sequences in clinical specimen, using DNA microarrays, is an alternative. Microarrays were first used as a diagnostic tool in 1993, to identify a hantavirus associated with an outbreak of acute respiratory diseases. The main advantage of microarrays is multiplexing, enabling exploration of the microbiota and pathogen detection in bacteremia, respiratory infections, and digestive infections: circumstance in which DNA arrays may lack sensitivity and provide false negatives. Enrichment of sampling can increase sensitivity. Furthermore, chips allow typing *Streptococcus pneumoniae* and detecting resistance in *Staphylococcus aureus* (MRSA) and *Mycobacterium tuberculosis* (rifampicin, isoniazid, fluoroquinolones). However, the cost and high technical requirements remain a problem for routine use of this bacterial infection diagnostic technology.

Keywords: Diagnosis; DNA microarray; Infectious diseases

Résumé

Le diagnostic des infections bactériennes repose sur l'isolement du pathogène, qui est rarement réalisé dans le temps du soin. La culture est mal adaptée au diagnostic des infections polymicrobiennes. Enfin, une approche syndromique doit cibler en parallèle les bactéries et les virus responsables d'un même syndrome. Une alternative est la détection de séquences ADN spécifiques dans l'échantillon clinique par les puces à ADN, dont la première application en 1993 était l'identification d'un hantavirus associé à une épidémie de maladies respiratoires. L'avantage essentiel des puces à ADN est le multiplexage, permettant l'exploration des microbiotes et la détection des pathogènes au cours des bactériémies, des infections respiratoires et des infections digestives, situation dans laquelle les puces à ADN peuvent manquer de sensibilité et donner des faux-négatifs. Une étape d'enrichissement du prélèvement peut palier cette limite. Également, les puces permettent le typage de *Streptococcus pneumoniae* et la détection de la résistance chez *Staphylococcus aureus* (méthicilline) et *Mycobacterium tuberculosis* (rifampicine, izoniaside, fluoroquinolones). Le coût et la technicité demeurent deux freins au déploiement en routine de cette technologie pour le diagnostic des infections bactériennes.

Mots clés : Diagnostic ; Infections ; Puces à ADN

1. Introduction

The diagnosis of bacterial infections in the laboratory relies on isolation of the bacterium, its identification, and antibiograms. This direct diagnostic approach is limited by: the delay before obtaining culture results, sometimes after caregiving; the presence of non-cultivable bacteria in the sample because of the presence of inhibitors such as antibiotics or because of inappropriate culture conditions; and the capacity to isolate and differentiate the various colonies of a polymicrobial sample. For example, around 75% of bacterial species in the digestive microbiotium, cannot be identified routinely with

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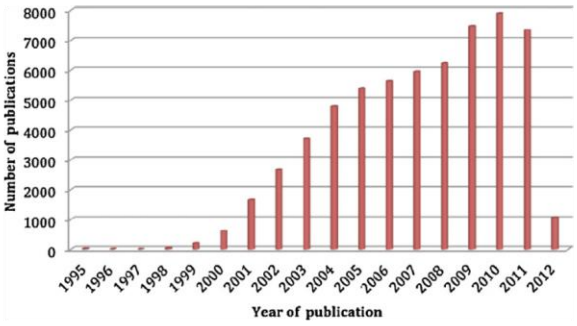

Fig. 1. Histogram of "DNA microarray" topics published in the PubMed database from 1995 to 2012 (May 2012).
Histogramme des publications concernant le sujet « DNA microarray » parues dans la banque de données Pubmed de 1995 à 2012 (mai 2012).

culture techniques [1]. An alternative to this direct diagnostic is the detection de universal gene sequences 16S rRNA or *rpo*B [2,3] by PCR followed by hybridization of a fluorescent oligonucleotide probe, with real time PCR [4]. As an alternative, the product of PCR amplification may be hybridized on a solid base fixing a great number of oligonucleotide probes, using the DNA microarray technique which is reviewed in this article. Searching for "DNA microarray" in the NCBI search engine (http://www.ncbi.nlm.nih.gov/pubmed/) gives 60,630 references (May 2012), showing the importance of the molecular biology tool described for the first time in 1995 [5] (Fig. 1). The "DNA microarray and infectious diseases" combination, gives 721 references (May 2012); the first reported use of this tool, for the diagnosis of a hantavirus infection, was published in 1993 [6].

2. Principle of DNA microarrays

The DNA microarray technique was adapted from the Southern blot by replacing the nylon base by a glass or silicon slide allowing covalent fixation of several thousand oligonucleotide probes. The probes correspond to: universal genes 16S rRNA [2] or *rpoB* [3]; or to genus, species, or serotype specific genes; to genes coding for resistance to antibiotics; or to toxinic genes. The DNA microarray technique includes several steps, such as manual or automatic extraction of nucleic acids from the clinical sample, their amplification by PCR, the labeling of amplification products by a fluorescent cyanine (Cy3- or Cy5-dCTP): a monochrome labeling is less expensive but a two-colored one has a better reproducibility [7,8]; then hybridization of labeled nucleic acids for 24 to 50 hours on the DNA microarray which is later scanned to measure the specific probe/DNA interactions of the sample. These interactions are measured by fluorescence as numerical data (Fig. 2). The total time for the procedure is inferior to 4 hours, not including time for hybridization.

There are few DNA microarrays available on the market for the diagnosis of bacterial infections. Two firms, Agilent (http://www.agilent.com) and Affymetrix (http://www.affymetrix.com/estore/), currently market custom made or prefabricated chips available in France. The Phylochip™ (Affymetrix; Santa Clara, California) chip carries 1.1 million probes for 25 nucleotides allowing the detection of the 16S rRNA gene for 60,000 bacterial species [9]. The CapitalBio Corporation firm (Beijing, China; http://www.capitalbio.com) markets a DNA chip for the identification and determination of resistance profile of 17 mycobacterial species, the most frequently isolated in laboratories. Finally, Prove-it™ Sepsis StripArray (Mobidiag, Helsinki, Finland) is an automated system coupling a PCR stage and a DNA microarray analysis for the detection of bacteria responsible for bacteremia.

3. Study of the microbiota

The human body carries interacting microorganisms called microbiota, containing ten times more cells than the human body and 100 times more genes than the human genome [10]. The DNA Phylochip™ (Affymetrix) allows investigating the human microbiota [9] (Fig. 3). Among these, the intestinal microbiota counting 10^{11} to 10^{14} bacteria [11] has a major role in the individual's homeostasis and in some diseases [12]. Three teams studied the intestinal microbiota with DNA microarrays carrying the probes of 25 to 40 bases targeting the 16S rRNA gene and detecting from 500 to 1140 bacterial species [11,13,14]. These authors reported that the intestinal microbiota includes a part found in all individuals belonging to phyla *Actinobacteria*, *Firmicutes*, and *Bacteroidetes*, and a part specific to each individual [13]. DNA microarrays also allowed observing a decrease of some Firmicutes and an increase of *Enterococcus*, *Clostridium difficile*, *Escherichia coli*, *Shigella flexneri*, and *Listeria* spp. species in patients presenting with Crohn's disease compared to healthy individuals [14].

The oral microbiota was also investigated with DNA microarrays detecting the gene 16S rRNA with probes carrying 18 to 20 nucleotides [15,16]. The Human Oral Microbe Identification Microarray (HOMIM) allowed investigating explorer the oral microbiota in five patients presenting with oral cancer, five patients presenting with pre-cancerous oral lesions, and ten healthy controls [15]. The authors of the second study analyzed the oral microbiota in 74 children 3 to 18 years of age, in four different groups according to the development stage of their teeth: (1) milk teeth, (2) early mixed dentition, (3) late mixed dentition, and (4) final dentition. The results of the two studies prove that Firmicutes, Bacteroidetes, Proteobacteria, and Actinobacteria are the most prevalent phyla in the oral microbiota depending on the individual's dentition: there are more proteobacteria than bacteroidetes in the final dentition group, contrary to the 3 other groups. The prevalence of *Prevotella* increases with age [16]. Finally, *Porphyromonas catoniae* and *Neisseiria flavescens* are significantly correlated to the presence of carries [16].

4. Diagnosis of bacterial infections

4.1. Respiratory infections

Three DNA microarrays were designed for the diagnosis of respiratory infections. A chip targeting the variable regions of the *gyrB* and *parE* of the bacterial genes detects

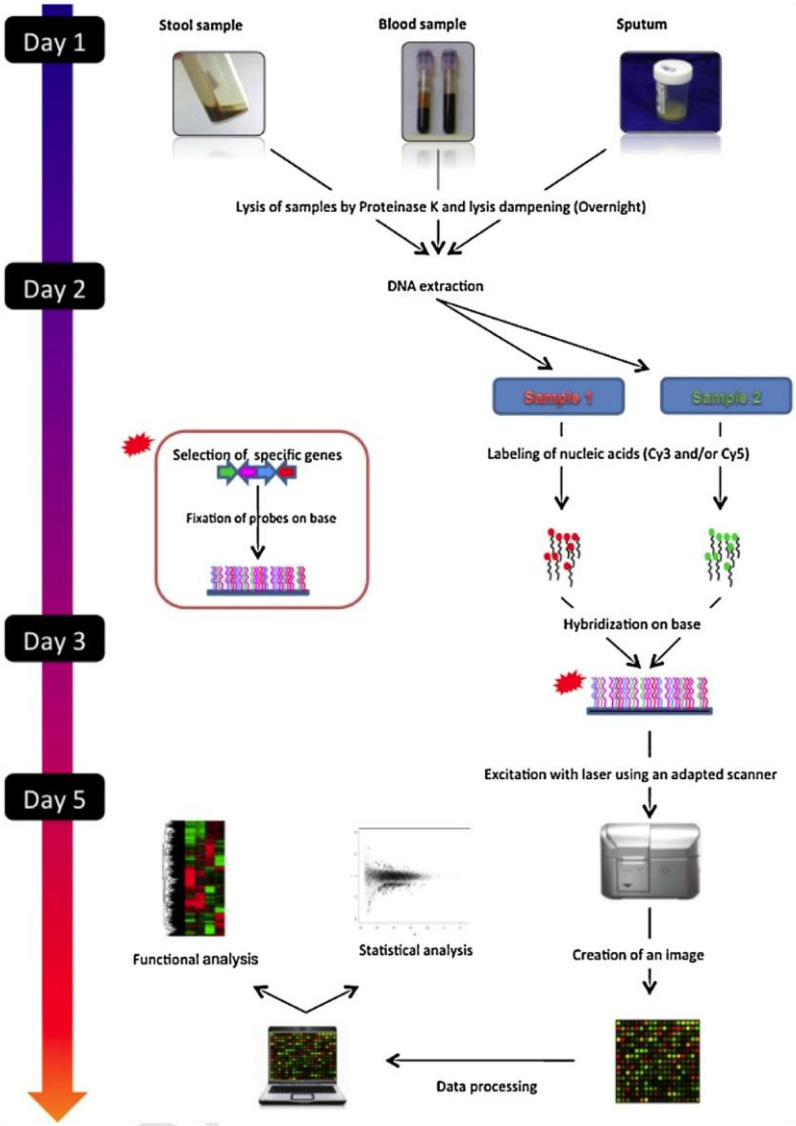

Fig. 2. Protocol for the DNA microarray analysis of clinical samples.
Protocole d'analyse des échantillons cliniques par puce à ADN.

Corynebacterium diphteriae, *Fusobacterium necrophorum*, *Haemophilus influenza*, *Legionella pneumophila*, *Moraxella catarrhalis*, *Mycoplasma pneumoniae*, *Staphylococcus aureus*, *Streptococcus pneumoniae* and *Streptococcus pyogenes*. Comparing with culture of 65 middle ear samples and 29 throat samples proved a better sensibility of the chip [17].

An other chip detecting Adenovirus, Bocavirus, Coronavirus type 229E, OC43, NL63, HKU1, Human Metapneumovirus types A and B, Influenza A-C, Para-influenza 1-4, Respiratory Syncytial Virus types A and B, Rhinovirus, *Chlamydia pneumoniae* and *M. pneumoniae* was used on 50 throat samples from adults during the winter 2007–2008 in Ireland [18]. The results proved that the chip gave a reliable diagnosis within one day. A third chip detecting 17 species of mycobacteria including *Mycobacterium tuberculosis* allowed directly analyzing 195 sputum samples from patients suspected to present with pulmonary tuberculosis, along with Ziehl staining and culture on agar [19]. Hundred and sixteen samples were found positive by the chip and 79 negative. The 116 positive samples were also positive in culture, but the chip identified a culture-negative

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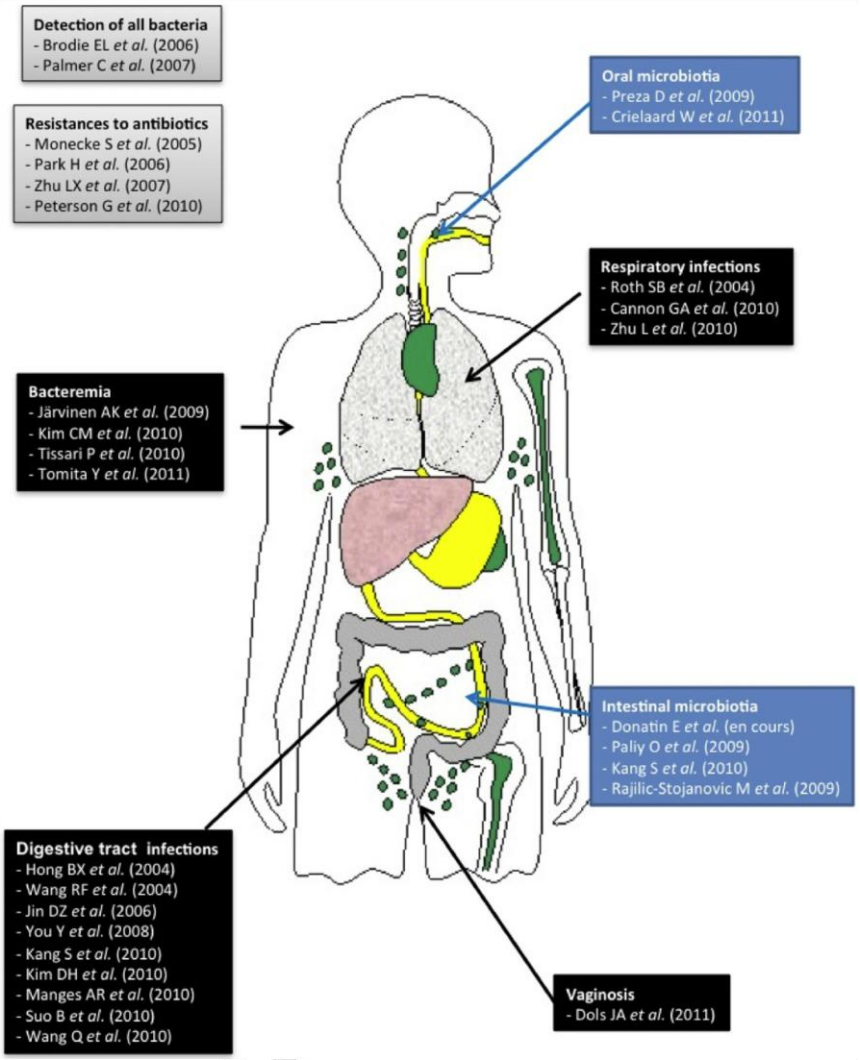

Fig. 3. DNA microarrays used to investigate various human microbiota, or for diagnostic purposes.
Les puces à ADN utilisées pour l'exploration des différents microbiotes humains, ou à visée diagnostique.

sample later. The chip detected *Mycobacterium intracellulare* in three patients confirmed by sequencing of the strains [19].

These various studies confirm the sensibility and specificity of the DNA microarray compatible with immediate use on respiratory tract samples.

4.2. Digestive infections

Two DNA microarrays target the variable regions of the bacterial ribosome sub-units 16S and 23S to identify 15 bacterial species frequently associated with food infections, such as *E. coli* O157:H7, *Salmonella enterica*, *Shigella dysenteriae*, and *Vibrio cholerae* [20,21]. A second kind of DNA microarray targets the virulence genes of enteropathogenic bacteria, for a combined detection of *E. coli*, *V. cholerae*, *Vibrio parahaemolyticus*, *S. enterica*, *Campylobacter jejuni*, *S. dysenteriae*, *S. flexneri*, *Shigella sonnei*, *Yersinia enterocolitica*, and *L. monocytogenes* [22]; or of enterotoxinogenic *E. coli* [ETEC], enterohemorrhagic *E. coli* [EHEC], *S. enterica* serovar Enteritidis, *S. enterica* serovar Typhimurium, *S. flexneri*, *S. sonnei*, *V. cholerae*, *V. parahaemolyticus*, *Vibrio vulnificus*, and *Y. enterocolitica* [23]; or of the 19 most frequent serogroups of enterotoxic *E. coli* [24]. A common limitation of all these chips is sensibility of detection. Indeed, some enteropathogens such as *Salmonella* spp. or *Campylobacter* spp. are presents in low inoculum less or equal to

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10^3 organisms/mL of diarrhea stools [25,26]. These inoculums are at the DNA microarray's threshold of detection [20,21], because of interference with some commensal species. A PCR amplification stage of pathogens before hybridization partly decreases this limitation [21].

One DNA microarray detects enteropathogenic bacteria *E. coli* O157:H7, *S. enterica*, *L. monocytogenes*, and *C. jejuni* in fresh food samples [27]. Twelve identical hybridization zones allow analyzing 12 different samples on a single slide so as to reduce test cost.

4.3. Bacteremia

Three DNA microarrays were described for the diagnosis of bacteremia. A chip targeting the bacterial gyrB and parE detects *Acinetobacter baumanii*, *Enterococcus faecalis*, *Enterococcus faecium*, *H. influenza*, *Klebsiella pneumoniae*, *L. monocytogenes*, *N. meningitidis*, *S. aureus*, *S. epidermidis*, *Streptococcus agalactiae*, *S. pneumoniae* and *S. pyogenes*, and the mecA gene associated with methicillin resistance in *S. aureus* [28].

It was tested on 146 positive hemoculture and 40 negative hemocultures, with a sensibility of 96% and a specificity of 98% [28].

Another DNA microarray targeting the internal transcribed spacer (ITS) region located between the ribosomal sub-units 16S and 23S, carries a universal positive probe control, two probes for the detection of Gram-positive bacteria, 1 probe for the detection of Gram-negative bacteria, nine genus specific probes (*Enterococcus*, *Listeria*, *Staphylococcus*, *Streptococcus*, *Bacteroides*, *Enterobacter/Klebsiella*, *Haemophilus*, *Pseudomonas* and *Serratia*) and 30 species specific probes [29]. Its sensibility was 85.8% on 825 blood samples within 1 hour [29]. The Prove-it^{TM} Sepsis StripArray includes PCR targeting the genes *gyrB* and *parE* to identify 50 Gram-positive and Gram-negative bacteria responsible for bacteremia, and the *mecA* gene associated with methicillin resistance in less than 3.5 hours [28,30]. It was tested on 3318 blood samples 63.5% of which were positive in culture, a proved a sensibility of 94.7% and a specificity of 98.8% [30]. The results of these studies prove that DNA microarrays are perfectly adapted for the diagnosis of bacteremia and the detection of methicillin resistance in *S. aureus* with a sensibility and a specificity of 100%, with faster results than standard methods relying on culture; indeed these two studies include a hybridization stage which lasts less than 1 hour.

4.4. Serotyping

A DNA microarray targeting the genes of glycosyltransferase (GT) [31] allows typing *S. pneumoniae* strains correlated with serotyping of this bacterium which includes 90 different serotypes [32]. Indeed, 25.6% of these serotypes are responsible for more than 90% of *S. pneumoniae* infections [33]. This chip, the first to use GT genes as molecular target, allows determining the serogroup of the *S. pneumoniae* strain in a single step.

4.5. Detection of resistance to antibiotics

4.5.1. Non-specific chip

Peterson et al. developed a DNA microarray allowing identification of 43 pathogenic bacteria (human and animal) targeting genes of resistance to antibiotics and genes of virulence [34]. The chip carries specific 227 probes for 90 bacterial genes of resistance, 99 probes targeting the genes of resistance to 20 metals, 113 specific probes for genes of virulence, 31 probes targeting transferable elements and seven probes corresponding to positive controls. The specificity of the chip was assessed on cultures of *S. enterica Typhimurium*, *Fusobacterium necrosum*, *E. faecalis*, and *E. coli* O157:H7. Little non-specific hybridizations was observed during the test. The tests made with this chip allowed confirming the results of previous studies with chips detecting only the *msrC* gene in strains of methicillin resistant *E. faecium* [35] and only the *pbp5* gene in strains of penicillin resistant *E. faecium* [36]. All the results obtained with the chip were confirmed by PCR.

4.5.2. Staphylococcus aureus

A DNA microarray was developed, detecting five specific *S. aureus* genes by targeting the gene 23S rRNA, 23 genes of resistance to antibiotics (macrolides, lincosamides, streptogramins, tetracyclin, cotrimoxazole, and aminoglycosides) and ten genes coding for toxins. The chip was tested by analyzing 100 methicillin resistant *S. aureus* strains [37]. A second chip identifying *S. aureus* versus *S.* non-*aureus* (gene 16S rRNA) and detecting the genes *mecA* and *blaZ* for methicillin and penicillin resistance, *aac*(6′)-Ie-*aph*(2″) for resistance to aminoglycosides, *ermA* and *ermC* for resistance to streptogramins, and *msrA* for resistance to macrolides, has a sensibility of 94.8 to 99% and a specificity of 69.3 to 99.2% according to molecular targets [38]. Nevertheless, some atypical results have been observed: some isolates have given signals of hybridization for the gene *blaZ* even though no β-lactamase activity was detected for these strains. One isolate, negative for the detection of the gene *blaZ*, had a β-lactamase activity. The authors explained this by a proved sequence variation at the gene *blaZ* level [37]. In this study, a decreased hybridization time from 90 to 30 minutes decreased the intensity of fluorescence by 10 to 30% [38].

4.5.3. Tuberculosis

M. tuberculosis resistance to rifampicin is related to the mutations in a region of 81 base pairs of the gene *rpoB* which codes for the RNA polymerase sub-unit β [39–43]. Isoniazid resistance is partly associated to a mutation of the gene *katG* which codes for a catalase-peroxidase and partly to a mutation of the regulator gene *inhA* [41,42]. A DNA microarray detecting these various mutations showed a sensibility of 93% and a specificity of 98.4% for the detection of rifampicin resistance; and a sensibility of 71.4% and a specificity of 97.6% for the detection of isoniazid resistance [43]. The authors recommend using chips to screen for multi-drug resistant (MDR) bacteria if tuberculosis is suspected.

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5. Conclusions

DNA microarrays for the diagnosis of infectious diseases were first described in 1993 [6] and have been the topic of a great number of publications (Fig. 1). Indeed, DNA microarrays allow a microbiological diagnosis within 48 hours (including hybridization time) compatible with the delay for medical management of patients. DNA microarrays also allow multiplexed detection adapted to a syndromic approach of infectious diseases diagnosis and to the diagnosis of polymicrobial infections extended to viruses. All authors agree that DNA microarrays have a sensibility and specificity at least equal to reference tests.

Nevertheless, designing the chip is a crucial step to obtain reliable results. The various chosen probes should have a very similar length and fusion temperature to optimize hybridization conditions, the current limiting stage. Several teams have solved the problem of cross-reaction by fixing, on the slide, specific probes for the same pathogen, in a redundant manner, and the current standard is a triplicate test. The sample preparation and the labeling of nucleic acids should be even more simplified for a routine use in bacteriological laboratories, to decrease time and cost of the test, and to integrate DNA microarrays among available techniques for the diagnostic point-of-care of infectious diseases [44]. Indeed, the currently available techniques, real time PCR and immunochromatographic methods are rapid diagnostic methods for a single pathogen per test. DNA microarrays have the advantage to allow multiplexed diagnosing perfectly adapted to a syndromic approach of infectious diseases diagnosis.

Disclosure of interest

The authors have not supplied their declaration of conflict of interest.

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CHAPITRE 2:

Optimized microbial DNA extraction

from diarrheic stools

Article 1–Préambule

L'utilisation des techniques de biologie moléculaire pour l'exploration du microbiote intestinal est limitée par la présence de nombreux inhibiteurs de PCR dans les selles comme les sels biliaires. En cas de diarrhée infectieuse, la dilution de la selle peut rendre la détection de certains pathogènes en faible inoculum difficile voire impossible. Notre travail étant basé sur l'exploration de la flore intestinale par des techniques de biologie moléculaire, nous avons tout d'abord optimisé les protocoles de préparation des selles diarrhéiques. Nous avons concentré les selles liquides en utilisant la lyophilisation. De plus nous avons mis au point un protocole d'extraction d'ADN qui combine une étape utilisant l'automate EZ1 à une étape manuelle d'extraction au phénol-chloroforme.

Optimized microbial DNA extraction from diarrheic stools

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Submitted to BMC Research Notes

Abstract

Background: The detection of enteropathogens in stool specimens increasingly relies on the detection of specific nucleic acid sequences. We observed that such detection was hampered in diarrheic stool specimens and we set-up an improved protocol combining lyophilization of stools prior to a semi-automated DNA extraction.

Findings: A total of 41 human diarrheic stool specimens comprising of 35 specimens negative for enteropathogens and six specimens positive for *Salmonella enterica* in culture, were prospectively studied. One 1-mL aliquot of each specimen was lyophilised and total DNA was extracted from lyophilised and non-lyophilised aliquots by combining automatic and phenol-chloroform DNA extraction. DNA was incorporated into real-time PCRs targeting the 16S rRNA gene of all bacteria and *Methanobrevibacter smithii* and the chorismate synthase gene of *S. enterica*. Whereas negative controls consisting in DNA-free water remained negative, *M. smithii* was detected in 26/41 (63.4%) non-lyophilised (Ct value 28.78±9.1) versus 39/41 (95.1%) lyophilised aliquots (Ct value 22.04±5.5); bacterial 16S rRNA was detected in 33/41 (80.5%) non-lyophilised (Ct value 28.11±5.9) versus 40/41 (97.6%) lyophilised aliquots (Ct value 24.94±6.6); and *S. enterica* was detected in 6/6 (100%) non-lyophilized and lyophilized aliquots (Ct value 26.98±4.55 and 26.16±4.97, respectively) and were negative for the 35 remaining diarrheal-stool specimens. The proportion of positive specimens was significantly higher after lyophilization for the detection of *M. smithii* ($p=0.00043$) and all bacteria ($p=0.015$).

Conclusion: Lyophilization of diarrheic stool specimens significantly increases the PCR-based detection of microorganisms. The semi-automated protocol described here could be routinely used for the molecular diagnosis of infectious diarrhea.

Keywords: DNA extraction, lyophilization, diarrheal stools

Findings

Infectious diarrhea is a leading cause of mortality and morbidity worldwide, being responsible for 2.16 million deaths a year, including 1.5 million pediatric deaths (3.7% of deaths in the world) (http://who.int/en/). Infectious diarrhea is caused by a wide spectrum of enteropathogens including the bacteria *Salmonella* spp., enteropathogenic *Escherichia coli*, *Shigella* spp., *Yersinia* spp., *Campylobacter* spp. and *Clostridium difficile* [1] and noroviruses, rotaviruses, toroviruses, coronaviruses, astroviruses, enteroviruses and adenoviruses, reportedly causing 50% of cases of diarrhea [2]. As most of these pathogens are fastidious to culture, the direct diagnosis of infectious diarrhea relies on the detection of enteropathogen-specific antigen by immunochromatographic assays [3-6] and the detection of enteropathogen-specific nucleic sequences by PCR, real-time PCR and DNA microarray [7,8]. Later detection however is hampered by the presence of PCR inhibitors [9] and the dilution of targeted pathogen in watery stools. When the normal excretion of water in stool is comprised between 150 and 200 mL every 24 hours [10], water excretion may increase up to one liter in diarrheic stools [11]. Previous studies showed that a preliminary enrichment step of stool increases the detection of enteropathogen DNA [12,13]. However, such an enrichment step delays molecular testing for up to 48 hours. We therefore aimed to optimize the DNA extraction protocol to target both bacteria and archaea in diarrheal stool specimens.

A total of 56 stool specimens (15 non-diarrheal specimens and 41 diarrheal stool) were prospectively collected in 56 individuals as part as the routine diagnostic activity in the Microbiology laboratory, Timone Hospital, Méditerranée Infection, Marseille, France. A total of 50 stools were negative for the routine detection of pathogenic bacteria and six diarrheal stool specimens yielded *Salmonella enterica* in culture. No written consent was needed for

this work in accordance with the “Loi n° 2004-800 relative à la bioéthique” published in the “Journal Officiel de la République Française” the 6 August 2004 since no additional sample was taken for the study. According to this law, patients were informed that stool specimens could be used for anonymised study. This study was approved by the local ethic committee of the Institut Fédératif de Recherche 48, Faculty of Medicine, Marseille, France under the reference number 08-002. Non-diarrheal and diarrheal stool specimens were treated separately.

In a first step, the 15 non-diarrheal stool specimens were diluted 1:5 in sterile phosphate buffer (PBS) in order to mimic diarrheal stool specimens. These 15 diluted specimens were divided into two aliquots, one-mL aliquot was frozen for 24 hours at -20°C and freeze-dried for 24 hours in one-mL glass containers (Dominique Dustcher, Brumath, France). After lyophilization, stool specimens were regenerated into 250 µL PBS resulting in a four-fold concentration of the stool specimens. The second aliquot was not lyophilized. The DNA extraction was then performed for the two aliquots using a semi-automated protocol combining the EZ1 Advanced XL extractor (Qiagen, Courtaboeuf, France) and a phenol-chloroform DNA extraction [14]. For the extraction protocol, a 250 µL-aliquot of the suspension was transferred into a sterile screw-cap Eppendorf tube containing 0.3 g of acid-washed beads (≤106 mm; Sigma, Saint-Quentin-Fallavier, France) and shaken in a FastPrep BIO 101 apparatus (Qbiogene, Strasbourg, France) at level 6.5 (full speed) for 180 s to achieve mechanical lysis. The supernatant was collected and incubated overnight at 56°C with 180 mL of lysis buffer and 25 µL of proteinase K (20 mg/mL) from the Qiagen EZ1® DNA Tissue kit. A 100 µL-volume total DNA was then extracted from 200 µL specimens using the Qiagen EZ1® DNA Tissue kit in the EZ1 Advanced XL extractor. A final step of phenol-chloroform extraction was performed. Negative controls consisting of sterile DNA-free water

were introduced at all steps and underwent the same extraction process that was used for the stool specimens. We analyzed specimens by real-time PCR with systems targeting a 128-bp portion of the 16S rRNA gene of *M. smithii* (Genbank accession number JQ346750), all bacteria 16S rRNA gene and a specific system targeting a 121-bp part of the chorismate synthase gene of *Salmonella enterica* (Table 1). These real-time PCR systems were designed in our laboratory using the Primer3 software (http://frodo.wi.mit.edu/). The specificity of primers and probes (100% coverage and 100% identity for the targeted pathogens) was tested using megaBlast against the nr-NCBI database (http://blast.ncbi.nlm.nih.gov/Blast.cgi). In-silico analyses indicated that the universal bacterial system recognized more than 18,000 bacterial species, but also 300 archeal species and less than 10 eukaryota. Whereas the *M. smithii* system forward primer of the system matches with other *Methanobrevibacter* species including *M. oralis*, *M. arboriphilus*, *M. ruminantum* or *M. wolinii*, reverser primer and the probe were found to be specific for *M. smithii*. The system targeting *S. enterica* was designed based on the complete genome of *S. enterica* subspecies *enterica* Typhimurium strain 798 (Genbank accession number CP003386). Primers and probe of this system were found to be specific for *S. enterica*, allowing the amplification of *S. enterica* serovars Enteritidis, Heidelberg, Typhimurium, Gallinarum, Paratyphi A and B, Saintpaul, Scwarzengrund, Dublin and Newport. Primers and probes were diluted to 20 pmol/µL and 25 pmol/µL respectively. PCR mixtures (20 µL) contained 10 µL Master Mix (Qiagen), 0.5 µL each primer and probe, 0.5 µL uracil-DNA-glycosylase (UDG) (Invitrogen-Life Technologies, Saint Aubin, France), four µL water and four µL DNA. Real-time PCRs incorporated two-min UDG decontamination at 50°C and ten-min denaturation at 95°C followed by 40 cycles of one second at 95°C, 35 seconds at 60°C and 45 seconds at 72°C. Each specific real-time PCR incorporated a positive and a negative control. The cut-off of positivity was set-up at a 38

cycle threshold (Ct). All specimens were tested in duplicate. *M. smithii* was detected in 11/15 (73.3%) non-lyophilized aliquots with a cycle threshold (Ct) value of 33.73±7.5 versus 14/15 (93.3%) lyophilized aliquots (Ct value of 24.38±7.3) (Table 2A). The universal system gave positive results for 13/15 (86.7%) non-lyophilized aliquots (Ct value of 30.85±5.9) versus 15/15 (100%) lyophilized aliquots (Ct value of 23.68±5.9) (Table 2B). All these 15 non-diarrheal specimens were negative for the specific detection of *S. enterica* before and after lyophilization. There is no significant difference before and after lyophilization for the detection of *M. smithii*, all bacteria and *S. enterica*.

In a second step, the 41 diarrheal stool specimens were treated as described above: stools specimens were divided into two aliquots, one aliquot was lyophilized before DNA extraction and the second aliquot was not lyophilized. Lyophilization, DNA extraction and real-time PCR protocols were performed as described above. *M. smithii* was detected in 26/41 (63.4%) non-lyophilized versus 39/41 (95.1%) lyophilized aliquots (Ct value 22.04±5.5) (Table 3A); bacterial 16S rRNA gene was detected in 33/41 (80.5%) non-lyophilized aliquots (Ct value 28.11±5.9) versus 40/41 (97.6%) lyophilized aliquots (Ct value 24.94±6.6) (Table 3B); and *S. enterica*-DNA detection was negative in 50/50 (100%) negative specimens and was detected in 6/6 (100%) non-lyophilized and lyophilized aliquots (Ct value of 26.98±4.55 and 26.16±4.97, respectively) (Table 3C). The proportion of positive specimens was significantly higher after lyophilization for the detection of *M. smithii* (p=0.00043) and all bacteria (p=0.015) but not for *S. enterica*. For positive specimens, this protocol increased Ct values of 6.9 Ct for the detection of *M. smithii*, 5.2 Ct for the detection of all bacteria and 0.8 Ct value for the specific detection of *S. enterica.*

Our results were validated by the fact that all of the negative controls remained negative in all of the experiments and results were duplicated. Dehydratation of stool has been shown to prevent DNA hydrolysis on human non-diarrheic fecal samples [15]. Previous studies also reported that lyophilization of pig and bovine stool specimens significantly improved the sensitivity of enteropathogenic bacteria detection with a 1.5- to 2-fold increase in DNA recovery compared to fresh stool specimens [16,17]. Data presented here showed that in human also, the lyophilization of stool specimens prior to DNA extraction increased the sensitivity of real-time PCR-based detection of archaeal and bacterial DNA. Indeed, the comparison between non-lyophilized and lyophilized aliquots showed a significant, average 14-fold increase in the the detection of *M. smithii* by real-time PCR; an average 10-fold increase in the detection of all bacteria.

Interestingly, the favorable effect of the lyophilization was more important for diarrheal stool specimens than for non-diarrheal specimens. Lyophilization could be especially useful for the molecular detection of enteropathogens which are in low-inoculum in human diarrheal stool specimens such as *Salmonella* which is present at 10^3 organisms/mL [18] and *Shigella* and *Vibrio cholerae* which are present at 10^1-10^2 organisms/mL [19,20]. In this work we targeted *M. smithii* and all bacteria that are present in high inoculums but also *S. enterica* that is a low-inoculum pathogen.

Previous studies showed the importance of using the acid-washed beads to lyses organisms with a thick cell wall such as *M. smithii* [21] and *Mycobacterium tuberculosis* [22]. The protocol reported here also incorporated mechanical lysis prior to DNA extraction; accordingly, *M. smithii* was detected in 93.3-95.1% specimens, a value consistent with the reported 95.7% prevalence of *M. smithii* in the general population in France [21].

The protocol here reported, combining lyophilization and a semi-automated DNA extraction, could be used for the routine detection of enteropathogen DNA in diarrheal stool specimens and the molecular diagnosis of infectious diarrhea [23].

Competing interests

The authors declare that they have no competing interests.

Author's contributions

ED performed analyses; ED and MD interpreted data and wrote the draft.

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Table 1: Real-time PCR systems used to test the efficiency of stool concentration by lyophilization.

Bacteria	Target	Sequence (5' – 3')	Length (bp)
Methanobrevibacter smithii	16S rRNA	CCGGGTATCTAATCCGGTTC	20
		CTCCCAGGGTAGAGGTGAAA	20
		CCGTCAGAATCGTTCCAGTCAG	22
All bacteria	16S rRNA	AGAGTTTGATCMTGGCTCAG	20
		TTACCGCGGCKGCTGGCAC	19
		CCAKACTCCTACGGGAGGCAGCAG	24
Salmonella enterica	Chorismate synthase	CAAGAAATACCTGGCGGAAA	20
		CGGGACAAAAGAACGGATTA	20
		GTTCGGCATCGAAATCCGCG	20

M = C or A

K = T, U or G

Table 2a: *M. smithii* real-time PCR detection in 15 non-diarrheal stool specimens diluted in 1:5 in sterile phosphate buffer, before and after lyophilization (Cycle threshold [Ct] value). NA, not amplified.

Sample n°	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
Without lyophilization	40.5	NA	38.93	36.43	26.38	NA	27.92	39.63	NA	22.27	33.43	43.61	38.53	NA	23.39
With lyophilization	34.39	34.74	33.93	16.97	16.84	11.72	20.16	30.24	19.76	23.11	22.65	NA	31.15	23.28	22.41

Table 2b: All bacteria real-time PCR detection with a universal system in 15 non-diarrheal stool specimens diluted in 1:5 in sterile phosphate buffer, before and after lyophilization (Ct value). NA, not amplified.

Sample n°	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
Without lyophilization	33.01	22.22	27.99	NA	NA	22.76	42.97	31.76	27.71	27.82	28.18	38.91	35.02	32.25	30.5
With lyophilization	15.61	24.24	21.70	17.41	25.19	16.78	22.02	25.94	20.58	21.47	21.24	27.43	37.34	33.91	24.28

Table 3a: *M. smithii* real-time PCR detection in 41 diarrheal stool specimens, before and after lyophilization (Ct value).

Sample n°	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30
Without lyophilization	NA	25.55	NA	18.87	39.33	36.94	37.84	37.69	31.95	38.51	39.22	40.32	24.18	36.14	NA
With lyophilization	38.98	18.82	35.20	16.29	25.62	23.21	26.54	28.51	24.41	23.96	25.2	23.67	23.65	23.98	17.5

Sample n°	31	32	33	34	35	36	37	38	39	40	41	42	43	44	45
Without lyophilization	34.83	NA	NA	33.91	NA	36.45	18.55	NA	37.05	22.02	39.05	19.28	33.16	19.25	NA
With lyophilization	21.53	22.47	19.34	27.19	18.30	27.48	21.69	24.49	24.14	20.53	23.88	19.18	13.92	19.27	24.44

Sample n°	46	47	48	49	50	51	52	53	54	55	56
Without lyophilization	NA	NA	35.67	22.78	NA	19.2	15.97	19.59	13.92	17.98	18.4
With lyophilization	18.08	29.99	NA	14.52	12.63	19.23	15.32	18.52	13.57	18.25	18.27

Table 3b: All bacteria real-time PCR detection with a universal system in 41 diarrheal stool specimens, before and after lyophilization (Ct value). NA, not amplified.

Sample n°	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30
Without lyophilization	NA	NA	30.54	NA	28.83	34.47	35.95	36.64	27.85	28.04	34.85	34.02	NA	30.14	26.82
With lyophilization	34.65	17.44	26.86	29.49	28.21	24.99	23.43	39.07	22.81	25.02	22.53	32.33	25.78	28.33	26.36

Sample n°	31	32	33	34	35	36	37	38	39	40	41	42	43	44	45
Without lyophilization	33.42	26.45	34.22	NA	34.63	30.36	28.49	26.98	33.14	23.75	NA	22.79	31.95	31.05	33.50
With lyophilization	21.18	22.03	21.79	26.01	21.84	24.4	29.74	23.24	28.85	21.29	33.43	19.31	21.41	32.68	22.23

Sample n°	46	47	48	49	50	51	52	53	54	55	56
Without lyophilization	29.24	NA	NA	24	23.21	23.05	17.59	19.94	14.65	18.33	18.58
With lyophilization	20.62	35.95	43.68	22.91	10.82	23.24	16.33	19.66	14.43	19.15	19

Table 3C. Positive real-time PCR detection of *S. enterica* in 6 diarrheal stool specimens, before and after lyophilization (Ct value).

Sample n°	51	52	53	54	55	56
Without lyophilization	31.65	28.14	26.35	18.39	29.23	28.09
With lyophilization	31.75	26.93	26.52	16.85	26.59	28.34

CHAPITRE 3:

A DNA microarray for the versatile diagnosis of infectious diarrhea

Article 2–Préambule

Le diagnostic direct des diarrhées infectieuses est rendu difficile du fait de la diversité des pathogènes pouvant être responsables de la pathologie, de la présence de nombreux inhibiteurs de PCR dans les selles et de la dilution du pathogène dans les selles diarrhéiques. Un test multiplexé de détection des bactéries et virus entéropathogènes n'existe pas. Nous avons mis au point une puce à ADN composée de sondes nucléotidiques de 60 paires de bases permettant l'identification de 33 bactéries et sept virus connus pour être responsables d'entérite aigüe chez l'homme. Nous avons choisi l'Archaea *Methanobrevibacter smithii* comme contrôle interne puisqu'il est détecté dans les selles chez 95,7% des individus [40].

A DNA microarray for the versatile diagnosis of infectious diarrhea

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SUMMARY

Several bacteria, viruses and parasites cause diarrhea as coinfecting pathogens. We designed a DNA microarray comprising 60-bp probes spotted 194 times for the multiplex detection of 33 enteropathogenic bacteria and seven enteropathogenic viruses, and the archaeon *Methanobrevibacter smithii* was used as an internal positive control. Nine pathogen-free stool specimens were used as negative controls. One of these control specimens was further spiked with *Salmonella enterica* as a positive control. The microarray was then tested with 40 pathological stool specimens, comprising *S. enterica* (n=30), *Campylobacter jejuni* (n=4), pathogenic *Escherichia coli* (n=2) and adenovirus (n=4). *M. smithii* was detected in 47/49 (95.9%) specimens, no pathogen was detected in negative controls and *S. enterica* was identified in the *S. enterica*-spiked positive control. The overall specificity was 100% and the overall sensitivity was 97.5% because one *S. enterica* sample was missed by the microarray. The multiplexed detection of *C. jejuni* spiked into an adenovirus-positive stool sample gave positive results, with fluorescence values of 14.3 and 9.1, respectively. These data indicate that using the protocol developed in this paper, the DNA array allows for the multiplexed detection of some enteropathogens in stool samples.

INTRODUCTION

Infectious diarrhea is estimated to be the fifth leading cause of death worldwide, with an estimated 2.16 million deaths a year, including 1.5 million pediatric deaths (http://who.int/en/). In France, diarrhea is estimated to generate approximately three million yearly visits to a general practitioner (1). Pathogens known to be responsible for diarrhea include the bacteria *Campylobacter* spp. *Salmonella* spp., *Clostridium difficile*, pathogenic *Escherichia coli*, *Shigella* spp. and *Yersinia enterocolitica* (2) (http://www.ecdc.europa.eu/en/Pages/home.aspx). Viruses, including noroviruses, rotaviruses, toroviruses, coronaviruses, astroviruses, enteroviruses and adenoviruses, reportedly cause 50% of cases of diarrhea (3). In particular, noroviruses are now the leading cause of diarrhea and enteritis outbreaks worldwide (4, 5).

Routinely, human enteropathogenic bacteria and viruses are searched for separately in different clinical laboratories in hospitals, but co-infections have been reported, particularly in developing countries (6-9). Therefore, a multiplex detection approach is warranted to speed diagnosis for the proper treatment and isolation of contagious patients. Additionally, such an approach would allow for the detection of clusters and epidemics. A DNA microarray is such a technology for the rapid multiplexed detection of microorganisms in clinical specimens (10-14). Accordingly, DNA microarrays have been used to investigate stool microbiota (15-19). However, the use of DNA microarrays for the identification of enteropathogenic bacteria in human diarrheal stool specimens has been rarely reported (10, 12, 13, 20-23). These microarrays detected a few bacterial pathogens, and few DNA microarrays allowed for the multiplexed detection of pathogens (10-14).

We therefore customized a DNA microarray for the multiplex detection of 40 bacterial and viral enteropathogens and the archaeon *Methanobrevibacter smithii* as an internal control, which should be positive in all cases (24).

MATERIALS AND METHODS

Stool specimens

Nine pathogen-free stool specimens with normal consistency collected from healthy individuals were used as negative controls in all experiments. The control of carriage *Staphylococcus aureus* in stool is mandatory for some workers in hospital under French law. These stools were used as "healthy individuals" without diarrhea. One of these control specimens was spiked with 10^8 colony-forming units (CFU)/mL (final concentration) *Salmonella enterica* CIP 60.62 serotype Typhimurium (Collection de l'Institut Pasteur, Paris, France) in phosphate-buffered saline (PBS) and used as a positive control. Human diarrheal stool specimens routinely submitted to the Méditerranée Infection clinical microbiology laboratory, Marseille, containing *S. enterica* (n=30), enteropathogenic *Escherichia coli* (EPEC) (n=1), enterohemorrhagic *E. coli* (EHEC) (n=1), *Campylobacter jejuni* (n=4) and adenoviruses (n=4) were collected. Bacteria were routinely detected by culture methods, as previously described (2). Caliciviruses and enteroviruses were routinely detected by a specific real-time PCR method using previously described primers (25, 26). Rotaviruses were detected by an immunochromatographic assay (Standard Diagnostics, Gurgaon Haryana, India). All of the viruses were further detected by electron microscopy observation. Among these 40 diarrheal stools, no stool specimen was co-infected. No written consent was needed for this

work in accordance with the "LOI n° 2004-800 relative à la bioéthique" published in the "Journal Officiel de la République Française" on August 6, 2004 because no additional sample was taken for the study. According to this law, patients were informed that stool specimens could be used for anonymized studies. This study was approved by the local ethics committee of the Institut Fédératif de Recherche 48, Faculty of Medicine, Marseille, France, reference number 08-002.

Microarray design

The archaeon *M. smithii* was used as an internal positive control, as we previously showed that it was detected in 95.7% of human stool specimens (24). To choose the 40 pathogens present on our DNA microarray we based on a recent review of infectious diarrhea (2). DNA probes were designed on the basis of the 16S rRNA gene sequence for 15 bacterial enteropathogens and specific gene sequences for an additional 13 bacterial enteropathogens as well as for viruses spotted on our microarray (Tables 1 and 2). Among these pathogens, we designed one specific probe for the detection of *Grimontia hollisae* that is responsible of human diarrhea for people who consumed raw shellfish, especially oysters or more rarely raw or undercooked fish (27), and *Klebsiella oxytoca* that is responsible of antibiotic-associated hemorrhagic colitis (AAHC) especially in children (28). There are unpublished internal evidences of the association between *Planctomycetes* and the intestinal microbiota (Drancourt M, 2012, unpublished data), that is why we designed specific probes for the detection of *Gemmata obscuriglobus, Pirellula staleyi, Planctomyces brasiliensis/maris, Planctomyces limnophilus* and *Rhodopirellula baltica.* In particular, five probes were spotted for the detection of pathogenic *E. coli*: for enterohemorrhagic *E. coli* (EHEC), we spotted *eae* and

stx1 gene probes (29); for enteroinvasive *E. coli* (EIEC), we spotted *ipaB* and *ipaD* gene probes (30); for enteropathogenic *E. coli* (EPEC), we spotted the *eae* gene probe (31); and for Shiga toxin-producing *E. coli* (STEC), we spotted *stx1* and *stx2* genes probes (32). The DNA microarray (Agilent Technologies, Massy, France) comprised eight hybridization arrays containing 15,744 features, each consisting of two interlaced rectangular grids of 96 rows at 0.073323-millimeter spacing by 82 columns at 0.127-millimeter spacing. Each 60-mer probe had an approximately 80°C hybridization temperature. Each probe was spotted 194 times on each hybridization array.

DNA extraction

Diarrheal stools were lyophilized before DNA extraction. Briefly, stool specimens were freeze-dried for 24 hours in 1-mL glass containers (Dominique Dustcher, Brumath, France) in the same lyophilizer with the negative control stool specimen. After lyophilization, stool specimens were regenerated in 250 µL PBS, resulting in a four-fold concentration of the diarrheal stool specimens. Lyophilized specimens were then manipulated in parallel with non-diarrheal stool specimens, which were not lyophilized. Instead, one gram of non-diarrheal stool specimen was diluted into 5 mL PBS and vortexed with 3-mm glass beads (Dominique Dustcher) for 30 seconds. In total, 250 µL of supernatant was collected to avoid fecal debris, and glass beads (size < 106 µm; Sigma Aldrich, Saint-Quentin Fallavier, France) were added to grind the specimen using the FastPrep® apparatus (MP Biomedicals, Illkirch, France) at 6.5 m/s for 90 seconds. This step was repeated once. A total of 25 µL of proteinase K (Qiagen, Courtaboeuf, France) and 180 µL of lysis buffer provided by the Nucleospin Tissue kit (Macherey Nagel, Hoerdt, France) were added before overnight incubation at 56°C. Next,

100 µL total DNA was extracted from 200 µL specimen using the EZ1 DNA Tissue kit (Qiagen, Courtaboeuf, France). Extracted DNA was further purified using a phenol-chloroform protocol (33). Each extracted specimen was analyzed with a Nanodrop 2000 Spectrophotometer (Thermo Scientific, Wilmington, USA) to evaluate DNA amounts. The non-diarrheal stool specimens were not lyophilized because we used lyophilization to concentrate diarrheal stool where the pathogens could be in low inoculums in order to avoid the dilution effect.

PCR and real-time PCR

In parallel with the DNA microarray experiment, each stool specimen was tested with real-time PCR for the specific detection of *S. enterica*, EHEC, EIEC, EPEC, STEC, adenovirus and *M. smithii*. Primers and probes (Table 3) were diluted to 20 pmol/µL and 25 pmol/µL, respectively. PCR mixtures (20 µL) contained 10 µL Master Mix (Qiagen), 0.5 µL each primer, 0.5 µL uracil-DNA-glycosylase (UDG) (Invitrogen-Life Technologies, Saint Aubin, France), 4 µL water and 4 µL DNA. Real-time PCR conditions included 2 min of UDG decontamination at 50°C and 10 min of denaturation at 95°C, followed by 40 cycles of one second at 95°C, 35 seconds at 60°C and 45 seconds at 72°C. Each specific real-time PCR assay included a positive and a negative control. The cut-off for positivity was established at 38 cycle threshold (Ct). All of the specimens were tested in duplicate. The extraction of *C. jejuni* was validated by classical PCR using two specific pairs of primers. The first pair targeted the *fla* gene (34), and the second one targeted the *wlaC* gene (35). These primer pairs were designed in our laboratory and generated 3,390- and 600-bp fragments, respectively. Each PCR was performed in a 25-µL mixture containing 2.5 µL of 10X buffer (Qiagen), 0.5

206 µL of each primer, 2.5 µL of deoxynucleotide triphosphate mix (Euromedex,
207 Souffelweyersheim, France), one unit of Hot Start (Qiagen), 10.8 µL water and 5 µL DNA.
208 PCR was performed under the following conditions: a 5-min denaturation at 95°C; 40 cycles
209 of 30 s at 95°C, two minutes at 60°C and one minute at 72°C; and a final extension step of 5
210 min at 72°C for the *fla* gene; and denaturation for five minutes at 95°C; 40 cycles of 30 s at
211 95°C, 30 s at 55°C, and one minute at 72°C; and a final extension step of five min at 72°C for
212 the *wlaC* gene.

213

214 DNA microarray assay

215 The Genomic DNA ULS Labeling Kit™ and the ULS-Cy™3 reagent were used according to
216 the supplier's instructions for an 8X15K microarray (Agilent Technologies). This protocol
217 allowed for labeling 10 µL of DNA. Hybridization was then performed according to the
218 Agilent protocol by adding 25 µL of reaction mixture [2 µL of Cot-1 DNA 1.0 mg/mL (Life
219 Technologies), 0.5 µL of Agilent 100X Blocking agent and 22.5 µL of Agilent 2X Hi-RPM
220 hybridization buffer] to each labeled DNA specimen. Specimens were then incubated at 95°C
221 for three minutes and 37°C for 30 minutes. In total, 11 µL of Agilent-CGHblock was added to
222 each specimen and hybridized in a total volume of 45 µL at 65°C for 40 hours. All of the
223 samples were hybridized in duplicate on our microarray. The background value was fixed at
224 four fluorescence units, and the positivity threshold was set at nine fluorescence units. A
225 positive detection was defined by over two-thirds of the specific probes exhibiting a
226 fluorescence value higher than nine. Fluorescence intensity values were expressed as the
227 mean of intensities measured for all homologous positive probes. All data were then

normalized using “R” software, available online at http://cran.r-project.org/doc/manuals/R-admin.htmL#Top.

Multiplexed detection

To test the capacity of the DNA microarray to simultaneously detect several pathogens in one stool specimen, we collected a stool sample that was naturally infected by adenovirus. An aliquot of this stool specimen was spiked with 10^4-10^6 CFU/mL (final concentration) *C. jejuni* CIP 70.2 in PBS.

RESULTS

PCR and real-time PCR

The DNA extraction protocol used in this paper yielded 41±28 ng/ml total DNA.

M. smithii DNA was detected in the nine negative control stool specimens (Ct mean value, 30.14), in the stool sample spiked with *S. enterica* (Ct value, 34.18) and in the 40 pathological stools (Ct values, 21.18 to 31.23).

S. enterica DNA was not detected in the negative control stool specimens, but it was detected in the stool sample spiked with *S. enterica* (Ct value, 19.46). Thirty *S. enterica*-infected diarrheal stools were lyophilized before DNA extraction. The real-time PCR detection of *S. enterica* was positive in all specimens, with Ct values between 24.31 and 29.47. *S. enterica* was not detected in the remaining ten pathological stool specimens.

Regarding pathogenic *E. coli*, none of the five targets were detected in the negative controls or the positive control. The *ipaB* gene was detected in one pathological stool infected with *C. jejuni* (Ct value, 32.61). One pathological stool sample infected with EPEC was positive for the *stx1* gene (Ct value, 22.78). The *stx2* gene was detected in two pathological stools infected with *C. jejuni* (Ct values, 29.08 and 33.40, respectively). The *ipaD* and *eae* genes were negative for all stool samples tested.

Four adenovirus-contaminated stool specimens yielded Ct values between 16.43 and 21.68; adenovirus was not detected in the other pathological stools or control stools.

Four *C. jejuni*-infected pathological stool specimens yielded positive results for *fla* and *wlaC* genes, while the negative and positive controls and the remaining pathological stool specimens were negative.

DNA microarray detection

The *M. smithii* internal control was detected in 47/49 (95.9%) stool specimens tested, with fluorescence signals between 9 and 14.5 units.

Twenty-nine of 30 (96.7%) *S. enterica*-infected pathological stool specimens yielded 194 positive *S. enterica*-specific probes, with fluorescence signals between 9 and 11.1; no other pathogen was detected in the 30 specimens, and *S. enterica* was not detected in the remaining stool specimens.

The pathological stool specimen infected with EPEC yielded 194 positive *stx1* gene probes, with a mean fluorescence value > 10 units. The pathological stool contaminated with

EHEC yielded 178 positive *eae* gene probes, with a mean fluorescence value of 10.4 units. The *ipaB* probe was positive in 13/47 (27.7%) remaining stools without a pathogenic *E. coli*. The nine control stools and the 38 remaining pathological stools were negative for all probes specific for pathogenic *E. coli*.

Four *C. jejuni*-contaminated pathological stool specimens yielded 132 positive probes, with a mean fluorescence of 9.1 in all specimens; the remaining specimens were negative for *C. jejuni*.

Four adenovirus-infected pathological stool specimens yielded positive detection, with fluorescence values between 9.1 and 10.9, and the remaining specimens were negative for adenovirus. One of these pathological stools infected with adenovirus and spiked with *C. jejuni* yielded a positive detection of 10^5 and 10^6 *C. jejuni* CFU/mL with fluorescence values of 14.3 for adenovirus and 11.9 and 12.1 for *C. jejuni*, respectively; the 10^4 CFU/mL inoculum was not detected.

DISCUSSION

The results here obtained in a clinical microbiology laboratory, were interpreted as valid because all of the negative controls remained negative in all of the experiments. Additionally, DNA microarray data were controlled in parallel with real-time PCR, including the detection of *M. smithii* DNA as an internal positive control. Indeed, we previously showed that this archaeal DNA was detected in 95.7% of individuals (24), making this archaeon a suitable target to control for total DNA extraction and the absence of PCR inhibition in extracted stool specimens. Detecting *M. smithii* DNA was further used to confirm that the dilution of

291 diarrheal stool specimens did not prevent the DNA-based detection of pathogens. In this
292 study, we lyophilized diarrheal stools as lyophilization has previously been used to suppress
293 PCR inhibition in animal stool specimens (36,37). We therefore recommend lyophilizing
294 diarrheal stool specimens before detecting enteropathogenic DNA.

295 The DNA microarray reported in this paper allowed for the simplex detection of
296 enteropathogens in stool, with a sensitivity of 97.5%. However, detecting pathogenic *E. coli*
297 was problematic. We designed probes based on published virulence genes reported to be
298 specific for each pathogenic *E. coli*. EIEC strains were detected with *ipaB* and *ipaD* probes
299 (30). *IpaB* is a gene encoding an invasion protein found in not only *E. coli* strains but also
300 *Shigella* and *Salmonella* strains. This gene is known to be specific for these strains (38), but
301 we found that our *ipaB* probe gave positive results for 13/47 (27.7%) stool samples tested.
302 This result may be due to a lack of specificity of the probe despite our favorable in silico
303 analysis. Alternatively, this observation could be explained by the fact that this gene is much
304 more ubiquitous than previously reported. For example, only 6% of genes are common
305 between all *E. coli* strains, which are called the core genome (39), and in fact, we do not
306 really know the virulence genes that can reliably identify strains of *E. coli.*

307 Intestinal infections could be caused by several pathogens at the same time, but the
308 simultaneous detection of enteric bacteria and viruses has never been performed using a DNA
309 microarray. Developing a protocol for the multiplex detection of human enteric pathogens
310 was challenging, but our data indicate that it is possible to achieve the multiplexed detection
311 of some enteropathogens.

312 Previously reported DNA microarrays allowed for the detection of only a few bacteria
313 (12, 13, 20). Regarding viruses, DNA chips have allowed for the detection of the rotaviruses

314 A group (40-43). In 2009, a DNA microarray was designed for the detection of common
315 foodborne viruses, including human rotaviruses (44); however, this system was not adapted
316 for the diagnosis of human acute enteritis. No DNA microarray has been published for the
317 dual detection of viral and bacterial enteropathogens.

318 Our data confirm the proof-of-concept of multiplex detection for enteric pathogens
319 using a DNA microarray. Further studies will aim to reduce the turn-over time, which was
320 three hours in this study. The DNA microarray technique is amenable to automation and could
321 be used for epidemiological studies and the selection of stool specimens devoid of any known
322 pathogen for further investigations using additional approaches. The cost of DNA microarray
323 remains a negative point since this technique in our laboratory is estimated at about 130 € per
324 sample. Additionally, a more complete version of the DNA microarray could be used for the
325 repertoire of the gut microbiota using the protocol developed in this study.

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457 **Table 1.** Probe sequences targeting bacteria.

	Bacteria	Sequences	TM (°C)	Length (bp)
Intestinal pathogens	*Aeromonas caviae*	TTGTATGGAT ACCTTTTTAG AACAATTAAA GTGTGGATTC GATCGCATTC GTTGATTTCT	80,4	60
	Arcobacter butzleri	ATATGAACTT CTGCATTCAC TGTTCCCATT TCTATTGCTT CAACTATACC AGTTATTTGG	79	60
	Campylobacter coli	TGTTCTTACT TCAAGAGATG GTAGAGGGAT TAAAATCACA GGTAGCATAG GTGTAGGAGC	79,5	60
	Campylobacter fetus	GAAACTACTC GCAAATTTTA AGGCTCAAAA ATGATAAACG CTAAACTCAT AGATCACATC TT	78,4	62
	Campylobacter jejuni	CGAAGGTATC ATCATAAGTT TAAATGCTTA TGCAACCATA CTAGGACAAG AAATCACACT CG	79,9	62
	Campylobacter upsaliensis	TAAGGGTAAT ATTATCGAGG AATTTGTAGA GGCAAGGCAA GATGGCGAAA CGATTC	81,6	56
	Enterohemorrhagic *Escherichia coli* (EHEC)	Refer to *eae* and *stx1* probes		
	Enteroinvasive *Escherichia coli* (EIEC)	(ipaB) GATTATCCGA ACTCGACCCA GATTCACCAG AAAATAAAAA ATTAAGACGG GGAGAAATAC	80,9	60
		(ipaD) TTATTACATT CAGCCCCGAA AGAAGCTGAG CTTGATGGAT ATGAAATGAT ATCTCATAGA	80,9	60
	Enteropathogenic *Escherichia coli* (EPEC)	(eae) CATGAAGACT ATATCTATAA CATCCACACA ATAAAAAACC CTCCGAAGAG GGGGAAGAGG	81	60
	Shiga toxin-producing *Escherichia coli* (STEC)	(stx1) ACAAATAATG TTTTTTATCG CTTTGCTGAT TTTTCACATG TTACCTTTCC TGGTACAACT	79,2	60
		(stx2) AAATACTTTC TACCGTTTTT CAGATTTTAC ACATATATCA GTGCCCGGTG TGACAACG	80,4	58
	Grimontia hollisae	AAGGTAATTA GAAGTGAAAT TATCAAGGAC GTTTATAACC AACCCCTTCA CCCTGGCC	81	58
	Klebsiella oxytoca	ACTTATCACT CTCAAGGAAT CAGAAATGAT AAAAAGTTCG TGGCGTAAAA TTGCAATGCT	81,1	60
	Laribacter hongkongensis	GAACTGGGCT CTGGAAGAGT AAGCTGCATA TTTGTGGTAT ACAAATATAT CGTTGTTTTA	78,8	60
	Listeria monocytogenes	AGCATCCATT TACATTACAT AAAAAGGGGG GGTACTAGTG CAATCAATTG AAGACATCTG	81	60
	Salmonella enterica	ACATGAACAA GTTTCGGAAT GTGATCAATT TAAAAATTTA TTGACTTAGG CGGGCAGATA	80,9	60
	Shigella sonnei	ATTTATATCG GCGTAATATT ATCAGTCGTT ATTATCTCAG GTACGGGATA TGGTAGATGC AC	78,3	62
	Tropheryma whipplei	TAGCCATCTT GCCTCTGTTA TGGATGATAT TGAGGTATAC GATGCAACAA AAAAGACTAT T	80,1	61
	Vibrio alginolyticus	TTGTTTGTTC TCTCATTCGT ATTATTTATT TCAAGTACAT CATGTCTTCT GGCTGGAGTT A	78,6	61
	Vibrio cholerae	AAGGTTCCTT TTTGTAGAGG TGGGGAAAAG TGCATGTTTC TCTTCTTATT CATAGCCAAT	81,3	60
	Vibrio parahaemolyticus	AAATCTCCAG AGTTTGTTAA AACCGTTCCA AAACGAGGCT ATCAACTCAT TTGTACTGTT	80,8	60
	Vibrio vulnificus	CTTAATAACA AAAATAGAAA TGTAGGACGC CTTACCCTAC TCTGCTGTTT GTTTGCGGC	80,7	59
	Yersinia enterocolitica	TTTTTTAGAA AAGGGACAGT TTGTACAAGT TTTCGGCCTA ACAATAAAAC CAAACAAGCC	80,4	60
Intestinal microbiota	*Gemmata obscuriglobus*	TAGATAGTAG ACCCAGATAT GGGTTTACTG TCGAAGTTAA AATGCTAAGT ACCCCGCCTG	80,2	60
	Pirellula staleyi	ATCCCTAGAT TCCCTAATTA TTGCATACTG AATCCATAGG TATGCAAGGC CAACCCAG	81,9	58
	Planctomyces brasiliensis/maris	AAGCGACTTT TTCAATCATT TTTGAAAGAG TTTTTTGCTT GCTGAGTGAA ACACTCG	81,9	57
	Planctomyces limnophilus	ATTTTCTCGA TAATACGCGG GTGATACGCG AAGAGTTTCT ACATACATTT ACCGAACT	80,7	58
	Rhodopirellula baltica	AAGAACCTTA TCCTAGACTT GACATGCTTG AGAATCCCTA TGAAAGTAGA GAGTGCCCTT	80,3	60
Internal control	*Methanobrevibacter smithii*	CCTCCAACAT TAAAAGGTCG TGAAACTTTA ACATGGCCAT CATGTATTAA ATAGAAAGGA	80	60

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460 **Table 2.** Probe sequences targeting viruses

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Viruses	Sequences	TM (°C)	Length (bp)
Adenovirus	AAAACAAAAC AAACTCCTTT GGACAAGCTC CCTATATAGG ACAAAAAATC ACCAATCAGG	80,9	60
Astrovirus	TTAAGCCTGG GAAGGTCATC TGTAGTGACA GTATAGTTGG GTTATCCTTT TGTGGCTT	81,4	58
Bocavirus	AATTGAGTAT TAAACCTATA TAAGCTGCTG CACTTCCTGA TTCAATCAGA CTGCATCC	79,3	58
Hepatitis A virus	TAATACTTCT ATGAAGAGAT GCTTTGGATA GGGTAACAGC GGCGGATATT GGTGAGTTAT	80,4	60
Norovirus	GGAGAAGCCT CACTCCATGG TGAAAAATTT TACAGGAAAA TATCTAGCAA AGTCATACAT	79,8	60
Rotavirus	AAAGGAATTG ATCAAAAGAT GAGAGTACTT AATGCTTGCT TTAGTGTGAA AAGAATACCA GG	78,7	62
Calicivirus	AACCACTCCC CAGGTAGCTC AAATGTTTAA ATTTTATTTC CTTAACTGTG ATGCCACAC	81,3	59

463 **Table 3.** Real-Time PCR system use for the specific detection of *Salmonella enteric*, *Escherichia coli*, adenovirus and *Methanobrevibacter*

464 *smithii*.

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Micro-organisms	Sequences	Length (bp)
Salmonella enterica	CAAGAAATACCTGGCGGAAA	20
	CGGGACAAAAGAACGGATTA	20
	GTTCGGCATCGAAATCCGCG	20
Escherichia coli	GCTGCGCGTGCAAATGCG	18
	CATGGTCATCGCTTCGGTCT	20
	CATCAGAAACTGAACACCAC	20
Methanobrevibacter smithii	GCGCGAACCGGATTAGATAC	20
	GCGACCGTACTTCCCAGG	18
	CGATGCGGACTTGGTGTTGGGG	22
Adenovirus	GCCACGGTGGGGTTTCTAAACTT	23
	GCCCCAGTGGTCTTACATGCACATC	25
	TGCACCAGACCCGGGCTCAGGTACTCCGA	29

CHAPITRE 4:

Enteric viruses decrease the load of gut aerobic bacteria

Article 3–Préambule

Le microbiote intestinal est composé de 10^{11} à 10^{14} bactéries/g de selles [2]. La majorité de ces bactéries sont anaérobies. Les modifications de cette flore sont à l'origine de nombreuses maladies et notamment des diarrhées infectieuses. Nous sommes partis du principe qu'une bactérie peut adopter deux mécanismes de virulence pour être pathogène. Le premier mécanisme consiste à produire des toxines, c'est le cas des bactéries *Clostridium difficile* ou *Shigella dysenteriae*. Le deuxième mode d'action est de se multiplier de façon excessive au sein du microbiote. C'est ce deuxième mécanisme qui nous a intéressé et nous avons cherché à étudier les bactéries présentes à des concentrations supérieures à 10^{10} UFC/g de selle dans des selles collectées au laboratoire hospitalier de la Timone, Marseille, entre avril 2010 et avril 2011. Lors de l'analyse d'une selle dans un laboratoire hospitalier, les résultats de bactériologie et de virologie ne sont quasiment jamais confrontés. Nous avons donc comparés nos résultats avec les résultats obtenus par le laboratoire de virologie sur les mêmes échantillons. Nous avons collecté 664 selles (347 selles non-diarrhéiques et 317 selles diarrhéiques). Nous n'avons pas observé de différences significatives entre la composition bactérienne des deux groupes, avec 42 espèces bactériennes identifiées pour le groupe des selles non-diarrhéiques et 45 espèces bactériennes pour le groupe des selles diarrhéiques. Ce travail a permis de réfuter notre hypothèse de départ qui était que les pathogènes entériques présentent des doses infectantes élevées. En effet, la plupart des bactéries pathogènes identifiées par le laboratoire de bactériologie de l'hôpital de la Timone, n'ont pas été retrouvées par notre protocole de dilution. Enfin, nous avons pu constater une corrélation négative entre la présence d'un rotavirus ou d'un adénovirus et la concentration de la flore bactérienne intestinale.

Enteric viruses decrease the load of gut aerobic bacteria

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ABSTRACT

In this study, we search for enteric viruses in parallel to bacteria. The work was conducted between April 2010 and April 2011 at the Timone hospital in Marseille. During this time we collected 664 stools comprising of 347 non-diarrheal stools and 317 diarrheal stools. A dilution protocol was developed to study the bacterial flora in the faeces at concentrations above 10^{10} colony forming-units (CFU). We did not found any significant differences between the two groups. Regarding non-diarrheal stools, 131 (37.7%) stools were negative and 216 specimens (62.3%) were positive with at least one bacterium detected at the dilution 10^{-10}. For the diarrheal stools group, 121 (38.2%) were negative, and 196 (61.8%) were positive. We have grown 42 different bacteria species in the non-diarrheal group and 45 for the diarrheal group with no major difference between the two groups. We observed that the presence of an enteric virus in a stool had a negative correlation on the bacterial microflora. To determined if this diminution of bacteria in a stool in presence of a virus is significant, we used the *Khi-2* statistical test. Through this test, we could conclude that Adenoviruses, Caliciviruses and Rotaviruses have a negative impact on the bacterial population. We decided to observe the effects of these viruses on the four most common bacteria found in faeces in our study, i.e., *Escherichia coli*, *Klebsiella pneumoniae*, *Enterococcus faecalis* and *Enterococcus faecium*. *E. coli* is the bacterium most affected because three enteric viruses are lowers this population with *p-values* well below 5%. Regarding *K. pneumoniae*, results are the same as for *E. coli* except for Caliciviruses, which have no effects on this bacterium. At the opposite, we found no significant correlation between the *Enterococcus* inoculum and the presence of viruses. Rotaviruses alone have the ability to decrease the growth of *Enterococcus faecalis*. These results indicate interferences between enteric viruses and bacteria by an unknown mechanism.

INTRODUCTION:

In 2008, the World Health Organization classified diarrhoeal infections as the fifth leading cause of death worldwide, with 2.16 millions death per year (3.7% of death in the world) (http://who.int/en/). Diarrheal infections are responsible for approximately 1.5 million infants and children annual deaths. Diarrhea may be caused by viruses including rotavirus, calicivirus responsible of about 60% of viral diarrhea [1] with Norovirus; bacteria including *Salmonella* spp., *Escherichia coli* pathotypes, *Shigella* spp., *Yersinia* spp., *Campylobacter* and *Clostridium difficile* [2]; and parasites such as *Cryptosporidium* or *Giardia lamblia* [3]. Moreover, most vertebrates are colonized by several parasites at the same time [4]. The impact of these passing pathogens on resident microbiota is poorly known in part because of we use a disease-by-disease approach to study infectious diseases without taking account of interactions existing between the different micro-organisms responsible for these diseases [4]. This approach is certainly inappropriate when we know that parasitism is the most popular life-style [4]. Besides virus and parasites, enteropathogenic bacteria play a major role in infectious diarrhea in being preventable and curable by antibiotics. While several enteropathogenic bacteria secrete toxins responsible for diarrhea and extra intestinal manifestations, *Vibrio cholerae* is most famous toxin-producing bacterium with the production of the cholera toxin [5]. It is also the case for shiga toxin-producing *Escherichia coli* (STEC) [6], *Campylobacter jejuni* who produce the cytolethal distending toxin [7], or *Clostridium difficile* with the production of toxin A and toxin B [8]. Other proliferates into the intestine, eventually interacting with both the intestinal cells and the gut microbiota. Our work was based on this second virulence mechanism. We decided to establish a dilution protocol in order to explore the aerobic and cultivable bacterial flora. Previous studies report that the total number of cultivable microorganisms can surpass 10^{10} colony forming units (CFU)/g of stool

575 [9]. That is why we decided to dilute our stool and to study only bacteria with concentration
576 higher than 10^{10} CFU/g of stool.

577 In present work, we observed the relations between some resident bacteria with
578 concentrations $\geq 10^{10}$ CFU/g of stool, especially *Escherichia coli*, *Klebsiella pneumonia*,
579 *Enterococcus faecalis* and *Enterococcus faecium*, and enteric viruses in faeces

580

581 **MATERIALS AND METHODS**

582 **Stool collection.** Stool specimens were prospectively collected between April 2010 and April
583 2011, in the Microbiology laboratory, Timone Hospital, Méditerranée Infection, Marseille,
584 France. No written consent was needed for this work in accordance with the "LOI n° 2004-
585 800 relative à la bioéthique" published in the "Journal Officiel de la République Française"
586 the 6 August 2004 since no additional sample was taken for the study. According to this law,
587 patients were informed that stool specimens could be used for anonymised study. This study
588 was approved by the local ethic committee of the Institut Fédératif de Recherche 48, Faculty
589 of Medicine, Marseille, France under the reference number 08-002.

590

591 **Routine detection of bacterial and viral enteric pathogens.** Routine laboratory
592 investigations of stools included the isolation of *E. coli*, *Shigella* spp., *Salmonella* spp.,
593 *Yersinia* spp., *Campylobacter* spp. by inoculating the stool specimen on Hektoen medium
594 (Dominique Dutscher) incubated at 37°C for 24 hours to search for *Salmonella* and *Shigella*; a
595 Karmali medium (Oxoid, Dardilly, France), as previously described [10] at 37°C in micro-
596 aerophilic atmosphere for five days for *Campylobacter* spp.; a *Yersinia* selective agar (CIN
597 agar) (BD, Le Pont de Claix, France) incubated at 30°C for five days. Only for children under
598 two years, a Chapman (Oxoid) and a BCP (BromoCresol Purple) medium (Biomérieux,
599 Craponne, France) are seeding and incubated at 30°C for 24 hours. *Clostridium difficile*

research is also conducted in our laboratory by using the Wampole^{TM} Tox A/B Quik Chek kit (Inversness Medical, Princeton, USA). It is a rapid membrane enzyme immunoassay for the simultaneous detection of *C. difficile* glutamate dehydrogenase antigen and Toxins A and B. Caliciviruses and Enteroviruses are revealed by a specific real-time PCR method incorporating previously described primers and probe [11,12]. Rotaviruses are detected by an immunochromatographic assay for the detection of Group A rotavirus (Standard Diagnostics, Suwon, Korea). The test utilizes two antibodies in a solid phase sandwich immunochromatography to detect group specific proteins, including the major inner capsid protein, present in Group A rotaviruses. All viruses are also detected in stool specimens by electron microscopy observation as previously described [13].

Cultivation and dilution protocol. One milliliter of diarrheal stools and two grams of control stools of normal consistency were frozen at -20°C before used. One milliliter of diarrheal stool and one gram of non-diarrheal stool were diluted into nine milliliters of sterile phosphate buffered saline (PBS) and homogenized by shaking with sterile glass beads (Dominique Dutscher, Brumath, France) in order to separate cells from debris. Nine serial 1:10 dilutions in PBS were realized up to a final dilution of 10^{-10}. A 100-µL aliquot of the 10^{-10} dilution was spread on COS medium (5% sheep blood in a Columbia agar base; BioMérieux, Craponne, France) by using ten sterile glass beads. Inoculated plates were incubated at 37°C overnight. Colonies were counted using the ImageJ software (http://rsb.info.nih.gov/ij/). To ensure that our protocol did not include a bias due to the sampling method, we tested the location of the sample taken on the stool, i.e., inside only, outside only or a slice including inside and outside of the stool. Later study was performed on ten different stool specimens tested with the protocol described above.

Bacterial identification. We used Matrix Assisted Laser Desorption-Ionization Time-of-Flight Mass Spectrometry (MALDI-TOF MS) Autoflex spectrometer for a rapid identification of each colony, as previously described [14]. Briefly, one colony was deposit on a MALDI-TOF MS plate with 1.5µl of matrix [saturated solution of *a*-HCCA (alpha-cyano-4-hydroxycinnamic acid) in 50% ACN and 2.5% TFA. The test was performed in triplicate for each colony. We considered that an isolate was correctly identified by MALDI-TOF when the three spectra had a score ≥1.9.

Statistical analyses. Statistical analyses were done using the *Khi*^{2} test (Epiinfo V.6.0, http://wwwn.cdc.gov/epiinfo/). A p-value < 5%, was considered as indicative of a significant result. We used Mantel-Haenszel test for the majority of our analyses but in some cases, when the sample was smaller, we used the Fisher Exact test.

RESULTS

Routine detection of enteropathogenic microorganisms. A total of 664 stool specimens prospectively studied over a 12-month period included 347 non-diarrheal specimens and 317 diarrheal-stool specimens. Age distribution (two years to 94 years for non-diarrheal specimens and one month to 87 years for diarrheal specimens) and sex ratio (199 men and 148 women for the control group and 178 men and 139 women for the diarrheal group) did not significantly differ between the two groups. Routine laboratory investigation found a total of 48 enteropathogenic bacteria among 317 specimens (15.1%) including Rotaviruses (n=186), Adenoviruses (n=26) and Caliciviruses (n=20), *Salmonella* sp. (n=14), *C. difficile* (n=20), *S. aureus* (n=9), *E. coli* (O114, n=2; O86, n=1; O55, n=2), in diarrheal stool. No co-infection was reported.

Detection of bacteria at high concentration. Preliminary study of ten non-diarrheal specimens showed no significant difference between the three different methods, as the same bacteria were found each time with equivalent concentrations in the three conditions. This allowed us to validate our protocol and to be able to reliably interpret the results further obtained in our study. We observed that there was no significant difference between the composition of the two groups. Regarding the group of non-diarrheal stools, 131 specimens (37.7%) were negative and 216 specimens (62.3%) were positive with at least one bacterium detected at the dilution 10^{-10}. We cultured 42 different bacterial species in the non-diarrheal group (Table 1). For the diarrheal group, 121 stools (38.2%) were negative and 196 (61.8%) were positive with at least one bacterium detected at this concentration. We cultured 45 different bacterial species in the diarrhea group (Table 2). Regardless of the presence of diarrhea, the four most common bacterial species found in stool in 10^{-10} dilution were *E. coli*, *K. pneumoniae*, *E. faecalis* and *E. faecium* in the two groups.

Correlations between pathogens. The *Khi2* test allowed us to see that in general the presence of an enteric virus in a stool, liquid or not, has a negative impact on the bacterial microflora (Table 3A). We wanted to know if a particular virus was responsible for this decrease of the bacterial population. The statistical results show that the decline of bacteria in a stool is not related to a specific virus (Table 3B), since the presence of either Adenoviruses, Caliciviruses or Rotaviruses negatively correlated with the detection of *E. coli* (Table 4A). As in the case of the general population, *E. coli* is sensitive to all tested enteric viruses. Indeed, the *E. coli* population is significantly reduced in association with a virus like Adenovirus, Calicivirus or Rotavirus (Table 4B). *K. pneumoniae* the second bacterium most often found by our culture protocol was also affected by the development of a virus in feces (Table 5A). Unlike the two previous cases, Caliciviruses have no effect on the detection of *K. pneumoniae*

675 (Table 5B). Finally, we focused on the gender *Enterococcus* and especially on the species *E.*
676 *faecalis* and *E. faecium*. These two species are also sensitive to the presence of a virus in a
677 stool (Table 6A and 7A) but after watching more detailed results, we observed than only
678 Rotaviruses have impact on the *E. faecalis* population (Table 6B). *E. faecium* population is
679 also reduced in the presence of an enteric virus, but our study did not identify the virus
680 responsible (Table 7B).
681
682 **Statistical analyses.** The principal component analysis of our results by Epiinfo software
683 showed that there is a significant correlation between the age of a patient and the type of the
684 virus ($p<0,0001$) (Fig 1). The age of the patient is also correlated with the consistency of the
685 stool ($p<0,0001$) and the type of bacteria found in the stool ($p=0,022$). The consistency is also
686 correlated with the type of virus in the stool ($p<0,0001$). Finally, there is a significant
687 correlation between the type of the virus and bacteria found in a stool ($p<0,0001$) (Table 8).
688 Regarding Rotaviruses, statistical analyses showed that infection with this type of virus is
689 more frequent for children under four years (Fig 2). Since 60,2% of people infected with a
690 Rotaviruses are aged less than 4 years.
691
692 **DISCUSSION**
693 Data herein presented were interpreted as authentic since our positive controls gave
694 the expected results and all the negative controls we introduced in each culture-based and
695 PCR-based experiment remained negative. Data were reproducible when specimens were
696 tested in triplicate for MALDI-TOF MS analysis and in duplicate for real-time PCR. We
697 observed reproducible results when testing external and internal portions of stool specimens
698 of normal consistency, excluding a bias in diarrheic stool.

699 The human gut microbiota is composed mainly of bacteria belonging to three different
700 phyla: *Firmicutes* (14-31%), *Bacteroidetes* (9-42%) and *Actinobacteria* (7-10%) [15]. Our
701 study gave different results concerning the composition of our two groups. Regarding the
702 non-diarrheal group we found 61.9% (26/42) *Proteobacteria*, 35.7% (15/42) *Firmicutes* and
703 2.4% (1/42) *Actinobacteria*. For the diarrheal group, we found 71.1% (32/45) *Proteobacteria*
704 and 28.9% (13/45) *Firmicutes*.
705 We based our study on the hypothesis that bacteria must be in high concentrations in
706 feces to be pathogenic and the comparison of our results with results of the Timone laboratory
707 of bacteriology shows us the contrary. Our protocol did not allow the identification of 7/14
708 (50%) *Salmonella* organisms probably because of their concentration were below 10^{10} in the
709 stools examined. It is the same for *S. aureus*. 336 strains of *E. coli* were identified with our
710 protocol against only five in the laboratory of bacteriology. This difference is explained by the
711 fact that *E. coli* is one the species most represented in the intestinal microbiota [16]. The
712 laboratory research only potentially pathogenic species, whereas our protocol does not
713 distinguish between pathotype and commensal species. Our study also allows us to observe
714 the intestinal microbiota in different conditions. It has long been considered that an infectious
715 disease was the result of the intrusion of a single microbe in a host. Nevertheless it is known
716 than most hosts are colonized with more than one parasite [17]. There are probably
717 interactions between these parasites, but they are poorly studied. More and more studies have
718 examined these interferences such as in the case of Spanish flu outbreak, which has been
719 shown that secondary bacterial infection had a major impact on mortality [18]. A recent work
720 show these interactions between cowpox virus, *Babesia microti*, *Bartonella* spp. and
721 *Anaplasma phagocytophilum* by collecting blood samples in 5981 field voles during five
722 years [17]. The human gastrointestinal microbiota that represents a complex ecosystem
723 composed of bacteria, archae, yeasts, filamentous fungi and viruses [19], and where

724 connections between these organisms must be high. The viral community is also present in the
725 intestinal microbiota. 1200 viral genotypes were identified in human feces with a density of
726 up to 10^9 virions per grams of dry material [20]. These viruses must also play an important
727 role in these interactions, but little is known about relations between bacteria and viruses in
728 the human gut. Indeed, the detection of pathogenic bacteria and enteric viruses is routinely
729 performed in different laboratories. The human gastrointestinal microbiota is an important
730 element of the human body, which is receiving increasing attention. It is a complex ecosystem
731 which that requires a lot of work to better understand how it works. During a long time,
732 everyone thought that every living organism of this microbiota evolved completely
733 independently and that an infectious diarrhea was the result of the action of only one bacteria
734 or one virus. However, more and more studies show that bacteria, viruses and other
735 microorganisms of the human gut interact. Our work is in the same perspective and highlights
736 the close relationships between bacteria and enteric viruses. It is important to better
737 understand these interactions in order to care for and treat infectious diarrhea, and also for
738 infectious disease in general. It could be interesting to develop a protocol in vitro that imitates
739 the conditions within the human gut and attempt to reproduce the relations between bacteria
740 and viruses.
741 This negative effect of enteric viruses on the bacterial microflora could be also explained by a
742 simple dilution effect. Knowing that an enteric virus induces severe diarrhea it is not
743 surprising to observe a decrease of the bacterial concentration in the gut microbiota. Indeed,
744 normal feces are emitted 1-2 times a day, they are homogeneous and molded, formed of 78%
745 of water and 22% of dry material and diarrhea is defined as an acceleration of transit with
746 emission of stools too liquid and too frequent (more than 250 g per day)
747 (http://www.who.int/fr/). So, this decrease is simply due to the dilution of the stool. In order
748 to neutralize this dilution effect we planned to lyophilize diarrheal stools. The team of Rapp D

749 *et al*. has shown that lyophilization concentrates the stool before performing real-time PCR
750 [21]. This technique is very useful to detect pathogens present in small quantities in a stool
751 and significantly improves the sensitivity ($p<0.001$) of the real-time PCR. Lyophilization has
752 also the advantage to protect DNA contains in fecal samples against hydrolytic damages and
753 enzymatic degradation [22]. It could be a good solution to concentrate the gut microflora in
754 order to explore this community in general, including pathogens present in small amounts in a
755 stool.
756

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817 **Table 1:** List of the 42 bacterial species cultured from the non-diarrheal stool specimens.

818

819

Species	Number
Escherichia coli	163
Enterococcus faecalis	60
Klebsiella pneumoniae	25
Enterococcus faecium	24
Enterobacter cloacae	14
Pseudomonas aeruginosa	12
Klebsiella oxytoca	11
Citrobacter freundii	8
Proteus mirabilis	7
Micrococcus luteus	6
Citrobacter amalonaticus	5
Citrobacter braakii	5
Staphylococcus epidermidis	5
Salmonella sp.	4
Enterococcus avium	3
Morganella morganii	3
Staphylococcus aureus	3
Bacillus cereus	2
Citrobacter sp.	2
Enterobacter aerogenes	2
Pseudomonas monteilii	2
Staphylococcus haemolyticus	2
Staphylococcus hominis	2
Stenotrophomonas maltophilia	2
Acinetobacter genomospecies	1
Acinetobacter hebiensis	1
Acinetobacter lowffii	1
Citrobacter koseri	1
Comamonas kerstersii	1
Enterobacter sp.	1
Enterococcus hirae	1
Enterococcus phoeniculicola	1
Enterococcus raffinosus	1
Hafnia alvei	1
Lactococcus lactis	1
Leclercia adecarboxylata	1
Paenibacillus glucanolyticus	1
Proteus vulgaris	1
Pseudomonas beteli	1
Shewenella putrefaciens	1
Staphylococcus warneri	1
Streptococcus agalactiae	1

820 **Table 2:** List of the 45 bacterial species cultured from the diarrheal stool specimens.

821

822

Species	Number
Escherichia coli	173
Enterococcus faecalis	38
Klebsiella pneumoniae	38
Enterococcus faecium	24
Citrobacter freundii	16
Proteus mirabilis	15
Pseudomonas aeruginosa	14
Enterobacter cloacae	13
Morganella morganii	12
Stenotrophomonas maltophila	6
Klebsiella oxytoca	5
Staphylococcus epidermidis	4
Citrobacter sp.	3
Hafnia alvei	3
Proteus vulgaris	3
Salmonella sp.	3
Streptococcus salivarius	3
Acinetobacter baumanii	2
Acinetobacter genomospecies	2
Acinetobacter grimontii	2
Acinetobacter sp.	2
Citrobacter amalonaticus	2
Citrobacter braakii	2
Citrobacter youngae	2
Comamonas testosteroni	2
Enterococcus casseliflavus	2
Providentia stuartii	2
Pseudomonas sp.	2
Serratia marcescens	2
Streptococcus sp.	2
Acinetobacter septicus	1
Aeromonas caviae	1
Candida albicans	1
Enterobacter kobei	1
Enterococcus avium	1
Enterococcus dispar	1
Enterococcus sp.	1
Providentia rettgeri	1
Pseudomonas geniculata	1
Pseudomonas hibiscicola	1
Rhizobium radiobacter	1
Staphylococcus aureus	1
Staphylococcus haemolyticus	1
Staphylococcus lugdunensis	1
Streptococcus gallolyticus	1

Table 3A: Statistical analysis of the effect of enteric viruses on the bacterial flora

		All viruses			
		+	-		
All bacteria	+	105	359	p<00000001	(Mantel-Haenszel test)
	-	118	84		

Table 3B: Detailed calculations for Rotavirus, Adenovirus and Calicivirus

		Rotavirus			
		+	-		
All bacteria	+	85	359	p=0,00339	Mantel-Haenszel test
	-	92	84		

		Adenovirus			
		+	-		
All bacteria	+	13	359	p=0,000148	Mantel-Haenszel test
	-	13	84		

		Calicivirus			
		+	-		
All bacteria	+	7	359	p=0,000016	Mantel-Haenszel test
	-	13	84		

Table 4A: Statistical analysis of the effect of enteric viruses on the *Escherichia coli* population

		All viruses			
		+	-		
E. coli	+	58	267	p<00000001	Mantel-Haenszel test
	-	165	176		

Table 4B: Detailed calculations for Rotavirus, Adenovirus and Calicivirus

		Rotavirus			
		+	-		
E. coli	+	48	267	p<00000001	Mantel-Haenszel test
	-	129	176		

		Adenovirus			
		+	-		
E. coli	+	6	267	p=0,000189	Mantel-Haenszel test
	-	20	176		

		Calicivirus			
		+	-		
E. coli	+	4	267	p=0,000354	Mantel-Haenszel test
	-	16	176		

Table 5A: Statistical analysis of the effect of enteric viruses on the *Klebsiella pneumoniae* population

		All viruses			
		+	-		
K. pneumoniae	+	16	62	p=0,0098	(Mantel-Haenszel test)
	-	207	381		

Table 5B: Detailed calculations for Rotavirus, Adenovirus and Calicivirus

		Rotavirus			
		+	-		
K. pneumoniae	+	10	62	p=0,00342	Mantel-Haenszel test
	-	167	381		

		Adenovirus			
		+	-		
K. pneumoniae	+	5	62	p=0,308	Fisher Exact test
	-	21	381		

		Calicivirus			
		+	-		
K. pneumoniae	+	1	62	p=0,216	Fisher Exact test
	-	19	381		

Table 6A: Statistical analysis of the effect of enteric viruses on the *Enterococcus faecalis* population

		All viruses			
		+	-		
E. faecalis	+	25	79	p=0,0264	Mantel-Haenszel test
	-	198	364		

Table 6B: Detailed calculations for Rotavirus, Adenovirus and Calicivirus

		Rotavirus			
		+	-		
E. faecalis	+	20	79	p=0,045	Mantel-Haenszel test
	-	157	364		

		Adenovirus			
		+	-		
E. faecalis	+	4	79	p=0,499	Fisher Exact test
	-	22	364		

		Calicivirus			
		+	-		
E. faecalis	+	1	79	p=0,111	Fisher Exact test
	-	19	364		

Table 7A: Statistical analysis of the effect of enteric viruses on the *Enterococcus faecium* population

		All viruses			
		+	-		
E. faecium	+	12	49	p=0,016	Mantel-Haenszel test
	-	211	394		

Table 7B: Detailed calculations for Rotavirus, Adenovirus and Calicivirus

		Rotavirus			
		+	-		
E. faecium	+	11	49	p=0,065	Mantel-Haenszel test
	-	166	394		

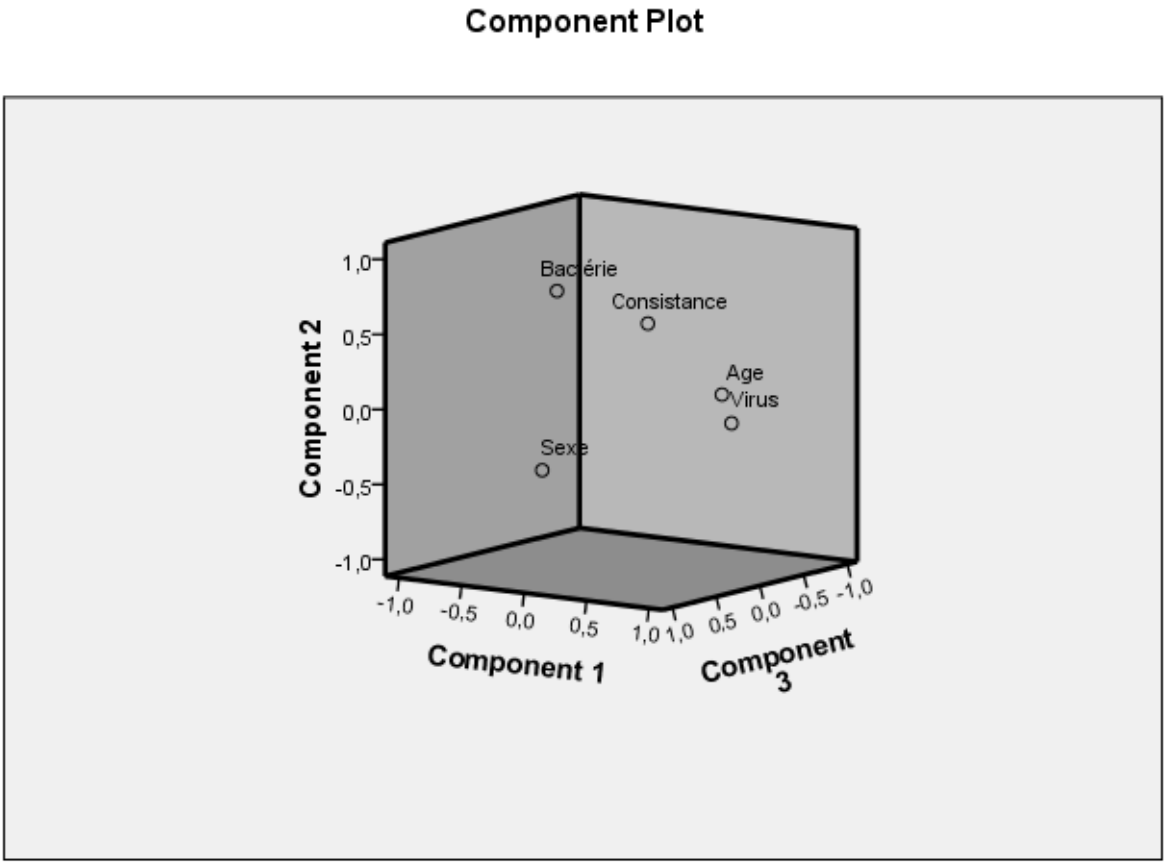
		Adenovirus			
		+	-		
E. faecium	+	1	49	p=0,21	Fisher Exact test
	-	25	394		

		Calicivirus			
		+	-		
E. faecium	+	0	49	p=0,101	Fisher Exact test
	-	20	394		

883 **Figure 1:** Principal component analysis of the correlation between the age of patients, the

884 sexe, the consistency of the stools, the virus and the bacteria found in the stool.

885



886

887

888 **Table 8:** Statistical analysis of the correlation between the age of patients, the sexe, the

889 consistency of the stools, the virus and the bacteria found in the stool.

890

Correlations

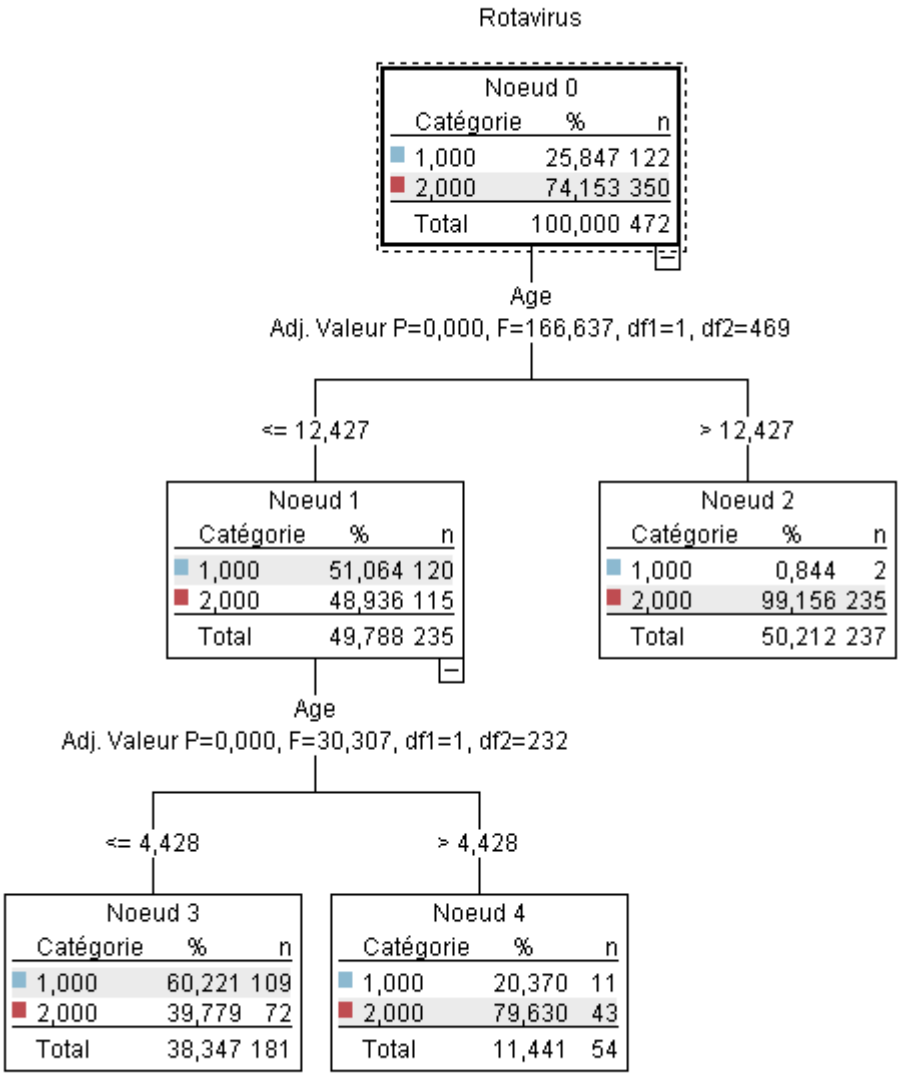
		Sexe	Age	Consistance	Virus	Bactérie
Sexe	Pearson Correlation	1	,003	,012	,005	-,019
	Sig. (2-tailed)		,931	,756	,895	,622
	N	664	663	664	664	664
Age	Pearson Correlation	,003	1	,166**	,498**	-,089*
	Sig. (2-tailed)	,931		**,000**	**,000**	**,022**
	N	663	663	663	663	663
Consistance	Pearson Correlation	,012	,166**	1	,158**	,004
	Sig. (2-tailed)	,756	**,000**		**,000**	,917
	N	664	663	664	664	664
Virus	Pearson Correlation	,005	,498**	,158**	1	-,221**
	Sig. (2-tailed)	,895	**,000**	**,000**		**,000**
	N	664	663	664	664	664
Bactérie	Pearson Correlation	-,019	-,089*	,004	-,221**	1
	Sig. (2-tailed)	,622	**,022**	,917	**,000**	
	N	664	663	664	664	664

**. Correlation is significant at the 0.01 level (2-tailed).

*. Correlation is significant at the 0.05 level (2-tailed).

891

Figure 2: Correlation between Rotaviruses and the age of the patient



1,000 mean presence of the bacteria or the virus

2,000 mean absence of the bacteria or the virus

Conclusion générale et perspectives

Le microbiote intestinal est un écosystème complexe où cohabitent bactéries, archées, virus et micro-eucaryotes. L'exploration de cette flore est d'autant plus complexe que tous ces microorganismes nécessitent des outils et des conditions d'identification différents. L'objectif de cette thèse était donc d'utiliser différentes approches afin d'explorer le microbiote intestinal et également de mettre au point un outil permettant une détection multiplexée des pathogènes responsables d'entérite aigüe chez l'homme.

Dans un premier temps nous avons étudié le microbiote intestinal grâce à une approche culturale qui consistait à observer les espèces bactériennes présentes dans les selles à des concentrations supérieures à 10^{10} UFC/g de selle [41]. L'étude s'est déroulée sur un groupe de 347 selles ayant une consistance normale et 317 selles diarrhéiques. La technique que nous avons mise au point ne permettait d'étudier qu'une partie de la flore bactérienne intestinale à savoir les bactéries aérobies cultivant à 37°C en 24 heures. Cette étude nous a permis de constater qu'il n'existe pas de différences significatives entre la composition bactérienne de ces deux groupes à de fortes concentrations. En effet, on retrouve sensiblement le même nombre d'espèces dans les selles normales (42 espèces différentes) ainsi que dans les selles pathologiques (45 espèces différentes). Ce travail nous a également permis de réfuter une de nos hypothèses de travail qui était qu'un pathogène doit se multiplier de manière excessive dans le tractus gastro-intestinal pour être responsable de diarrhée infectieuse. Or la plupart des pathogènes identifiés par le laboratoire de bactériologie de la Timone n'ont pas été mis en évidence par notre technique de dilution des selles. Ce premier travail nous ensuite amené à étudier les interactions existant entre les virus et les bactéries de la flore intestinale toujours à des concentrations supérieures à 10^{10} UFC/g de selle. Nous avons observé une diminution significative de la population bactérienne en présence d'un rotavirus ou d'un adénovirus.

Après avoir tenté, en vain, de comprendre le mécanisme d'action de ces virus sur la flore bactérienne, nous avons imaginé que cette diminution du nombre de bactéries pouvait être en partie liée à une simple dilution des selles. La présence d'une grande quantité d'eau dans les selles diarrhéiques à un effet diluant sur la flore bactérienne ce qui a pour conséquence que l'on retrouve moins de bactéries à des concentrations $\geq 10^{10}$ UFC/g de selle. Nous avons alors imaginé un protocole permettant de concentrer les selles diarrhéiques afin de faciliter leur étude par des techniques de culture mais également grâce à des outils de biologie moléculaire [42]. Pour cela nous avons utilisé la lyophilisation qui permet de retirer l'eau présente dans un échantillon de selle, et nous avons reconstitué ce même échantillon avec un volume de liquide plus faible afin de le concentrer. Nous avons amélioré cette technique de concentration des selles en la complétant par une étape d'extraction d'ADN semi-automatisée. La première partie de l'extraction est automatisée en utilisant l'automate EZ1 suivie par une étape manuelle utilisant la technique d'extraction au phénol-chloroforme. Cette technique nous a permis d'améliorer la détection par PCR en temps réel de notre contrôle interne *Methanobrevibater smithii* retrouvé dans les selles de 95,7% des individus [40] et de la flore bactérienne intestinale en général. La dernière partie du travail consistait à mettre au point une puce ADN pour le diagnostic multiplexe des diarrhées infectieuses. Cette puce consiste en des sondes oligonucléotidiques d'une longueur de 60 nucléotides permettant l'identification de 33 bactéries et sept virus intestinaux responsables d'entérite chez l'homme. Cette puce ADN nous a permis de détecter de façon reproductible la présence d'un seul pathogène dans une selle. Nous avons également réussi à mettre en place la détection simultanée d'un adénovirus et du pathogène *Campylobacter jejuni* présent dans une même selle. Cependant quelques améliorations sont à envisager avant de pouvoir utiliser cet outil pour le diagnostic des diarrhées infectieuses dans les laboratoires hospitaliers. La première amélioration serait de modifier le protocole d'extraction des acides nucléiques afin de pouvoir identifier les virus à

946 ARN tels que les rotavirus, les calicivirus ou le virus de l'hépatite A. Enfin la technique
947 d'identification par les puces à ADN étant relativement longue, il serait bon d'envisager une
948 automatisation du protocole afin de réduire le délai du diagnostic.
949

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