



Microbial forensics: new breakthroughs and future prospects

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Received: 2 August 2018 / Revised: 14 September 2018 / Accepted: 16 September 2018 / Published online: 9 October 2018
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Abstract

Recent advances in genetic data generation, through massive parallel sequencing (MPS), storage and analysis have fostered significant progresses in microbial forensics (or forensic microbiology). Initial applications in circumstances of biocrime, bioterrorism and epidemiology are now accompanied by the prospect of using microorganisms (i) as ancillary evidence in criminal cases; (ii) to clarify causes of death (e.g., drownings, toxicology, hospital-acquired infections, sudden infant death and shaken baby syndromes); (iii) to assist human identification (skin, hair and body fluid microbiomes); (iv) for geolocation (soil microbiome); and (v) to estimate postmortem interval (thanatomicrobiome and epinecrotic microbial community). When compared with classical microbiological methods, MPS offers a diverse range of advantages and alternative possibilities. However, prior to its implementation in the forensic context, critical efforts concerning the elaboration of standards and guidelines consolidated by the creation of robust and comprehensive reference databases must be undertaken.

Keywords Criminal investigation · Genetics · Massive parallel sequencing · Microbial forensics · Microbiome

Introduction

Microorganisms (e.g., viruses, bacteria and many fungi) can be found nearly everywhere on Earth, establishing remarkably diverse and ubiquitous communities. From the intersection of knowledge and expertise from microbiology and forensics, microbial forensics (MF or forensic microbiology) was born (Kuiper 2016). This discipline employs microbiological methods for analyzing evidence involved in a multitude of criminal cases classically aiming forensic attribution (assigning to a source), ranging from bioterrorism, biocrime, frauds, outbreaks and transmission of pathogens, or accidental release of a biological agent and/or a toxin (Allard et al. 2017; Amorim 2010; Budowle et al. 2014; González-Candelas 2017; González-Candelas et al. 2013).

Typically, pathogens' outbreak monitoring and toxicology are not considered as crucial elements of MF. However, these two topics are basic to MF, since pathogens' release can be intentional or due to medical malpractice and the application of a reliable and robust surveillance protocol for pathogen monitoring would provide valuable information to distinguish between spontaneous and harmful spread of microorganisms (either related with bioterrorist or biocrime). Besides the differences between protocols and objectives of forensic investigations and epidemiological studies, it is our opinion that the investigations of the sources of outbreaks to fall in the realm of MF due to the shared aim of origins' ascertainment.

When natural pathogens' outbreaks are concerned, measures to prevent other cases of the disease must be implemented. The success of such measures relies in the quantity and quality of the data collected on isolate identification (to the strain level), location of possible sources, definition of possible reservoirs, vectors and intermediary host, determination of transmission routes or evolution dynamics (Schürch and Siezen 2010). Over the preceding decades, the number of epidemic and pandemic has been increasing, examples of the latest cases of pathogens' outbreaks are (Arenas et al. 2017; Oliveira et al. 2018): *Mycobacterium tuberculosis* (tuberculosis; 2006, Canada; Gardy et al. 2011), *Acinetobacter baumannii* hospital outbreak (2008, United Kingdom; Lewis et al. 2010), swine-origin influenza A

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(H1N1 virus; 2009, America, 2009; Smith et al. 2009), *Vibrio cholerae* (cholera; 2010, Haiti; Hendriksen et al. 2011), the Middle East respiratory syndrome hospital outbreak (MERS coronavirus; 2012, Saudi Arabia; Assiri et al. 2013), avian-origin influenza A (H7N9 virus; 2013, China; Kageyama et al. 2013), Shiga toxin-producing *Escherichia coli* O104:H4 (2014, Germany; Grad et al. 2012), Ebola (Ebolavirus; 2014, Sierra Leone; Cenciarelli et al. 2015), poliomyelitis (Poliovirus; 2014, Middle East; Aylward and Alwan 2014), Legionnaires' disease (*Legionella pneumophila*; 2014, Portugal; Borges et al. 2016) and Zika virus (2015, Brazil; Faria et al. 2016).

In the case of a bioterrorist attack, a given biological agent (e.g., bacteria, viruses, fungi and toxins) is intentionally released with the purpose to cause panic, mass casualties or economic loss, being motivated by ideological, political or religious beliefs (Jansen et al. 2014). These agents can be used as they are present in nature or be genetically engineered for improved mass dissemination, more significant mortality or resistance to the currently available medicines and/or vaccines (Pavlin 1999). When facing the possibility of a bioterrorist attack is crucial to identify the agent involved, not only to prevent panic from the population but also to contain the morbidity and mortality associated with the dissemination of such agent (Lehman 2014). The emphasis of MF on bioterrorism has emerged from the challenges brought up by the responses to the famous Amerithrax mailing attacks in 2001 (Rasko et al. 2011). Besides *Bacillus anthracis*, other examples of agents used in bioterrorism attacks can be presented: *Yersinia pestis* (bubonic plague), *Salmonella enterica serovar* Typhi (typhoid fever), *Shigella dysenteriae* (dysentery), *Francisella tularensis* (tularemia), human immunodeficiency virus (HIV), Variola major and Variola minor viruses (smallpox), *Rickettsia prowazekii* (typhus), *Ascaris suum* (ascariasis); and toxins such as those produced by *Clostridium botulinum* (botulism), *Corynebacterium diphtheria* (diphtheria), *Staphylococcus aureus* [e.g., toxic shock syndrome (TSS), staphylococcal scalded skin syndrome (SSSS), necrotizing pneumonia, or deep-seated skin infections] and *V. cholerae* (cholera) (Jansen et al. 2014).

Biocrime can be defined as the weaponization of a disease-causing agent or toxin with the intention to harm or cause panic in a specific individual or in a limited group of individuals (Jansen et al. 2014; Lehman 2014). The use of MF in biocrime investigations can be illustrated by the paradigmatic cases of intended dissemination of HIV (Ciesielski et al. 1992; Kovach 2008; Metzker et al. 2002; Muchmore 2013), hepatitis B (HBV) (Muchmore 2013) and C (HCV) viruses (Akehurst 1998; Bosch 1998; Muchmore 2013). In such cases is of primordial importance to establish the source of the bioagent through the comparison of the isolate found in victims and that associated with the perpetrators (Lehman 2014).

Considering the topic of toxicology, if during an autopsy the microbiome present in a given organ from a cadaver is unveiled, the presence of bioindicator microorganisms can indicate the occurrence of postmortem production or degradation of several drugs or poison that otherwise would provide misleading information concerning the cause of death. Therefore, both aspects, outbreak monitoring and toxicology, will be addressed in the present review.

Since its introduction more than 30 years ago (Gill et al. 1985), forensic DNA testing has assumed a substantial role in civil and criminal cases (Butler 2015). DNA evidence can be used as a silent witness to establish a probability-based connection between a crime scene and particular individuals (suspects and/or victims) and/or objects. These links, determined by the comparison of the genetic profiles and complemented by other means of proof, are frequently decisive aiding in acquitting or convicting a suspect (Budowle et al. 2017; Butler 2015).

Previously to the advent of DNA-based techniques, in the late 1980s, the available tools for microorganism detection and identification were restricted to phenotypic methods associated with antigenic and/or antimicrobial resistance profiles. These methods only allow resolution at the genus and/or species level (using culture-dependent techniques) (Oliveira et al. 2018).

Afterwards, a paradigm shift was observed: the traditional phenotypic methods were supplanted by nucleic acid-based molecular methods [e.g., DNA fingerprinting, whole genome sequencing and microarray analysis] (Budowle et al., 2005; Pattnaik and Sekhar 2008). These methods not only improved taxonomical resolution (identification to isolate/strain level), being unsubjected to the changes observed phenotypic characteristics due to the influence of environmental factors, but also decreased the turnaround time between sample collection and results (Pattnaik and Sekhar 2008).

The next evolutionary step, in the early 2000s, was associated with the introduction of massive parallel sequencing (MPS) technology [also referred as next-generation sequencing (NGS) or high-throughput sequencing (HTS)] (Yang et al. 2014). Due to its increased multiplexing capacity, this method complies with different approaches, such as sequencing the whole genome (WGS) of a given microorganism (viruses, bacteria or fungi) or sequencing all the microbial species present in complex sample collected from a given individual or environment (metagenomics) (Kuiper 2016; Schmedes et al. 2016).

As such, presently, the forensic science has evolved and expanded, reaching out to numerous innovative technological breakthroughs and molecular advances. These recent developments have led to an increasing use MPS over conventional Sanger sequencing (Bano et al. 2015; Clarke et al. 2017; Schmedes et al. 2017). Such advances, like the ability to sequence single isolates with de novo assembly, read mapping,

targeted sequencing of specified genes or other regions of interest (e.g., hypervariable regions of 16S and 18S rRNA genes, 16S–23S rRNA intergenic spacer region, SNPs, indels) (Sijen 2015) and metagenomics (Børsting and Morling 2015; Franzosa et al. 2015), ensure a growing capacity of identification and characterization of microbial communities, paving the way for the use of microbial evidence in cases associated to human identification, geolocation and postmortem interval estimation, amongst other applications (Arenas et al. 2017; Kuiper 2016).

Although phylogenetic analyses have supported associations and have successfully been admitted as evidence in criminal cases, the fundamental limitations to the widespread application of MF are the non-existence of both standards and guidelines and the deficiency of reference databases, reference genome sequences, metadata and representative genetic diversity coverage (Arenas et al. 2017; Sjödin et al. 2013).

The main assets of MPS over classical Sanger sequencing are (i) its high-throughput capacity, hundreds of millions of sequencing reactions can be completed in parallel, permitting to sequence an entire bacterial genome in just one or two instrument runs; (ii) a unique protocol can be applied for all microorganisms for identification and genotyping; (iii) the necessity for DNA cloning is obviated, exclusively depending on libraries preparation, in a cell-free system; (iv) there is no need for an a priori knowledge about the sequence of a particular gene/genome because these new technologies can read the DNA templates randomly distributed throughout the entire genome, and then *de novo* genome assembly can be applied; (v) overcome the need for isolation and culture of the microorganism of interest, a particularly critical aspect once many strains are unable to grow in culture media, allowing to identify microorganisms present in trace concentrations and undetected by conventional methods; and (vi) reduces both costs (usually less than US\$1000 per genome, depending on the genome length) and the turnaround time (only a few hours) (Oliveira et al. 2018; Schmedes et al. 2016).

The main shortcoming of the MPS is associated with the shorter read length for each DNA template. Nowadays, read length ranges from 35 to 500 base pairs (bp) comparing with the 1000–1200 bp obtained with Sanger sequencing (Oliveira et al. 2018; Van Dijk et al. 2014). Furthermore, the application of these approaches poses two challenging tasks: data storage and analysis (van Dijk et al. 2014; Kuiper 2016). Depending on the platform used, 30,000–500,000 sequence reads of 50–700 nucleotides can be generated in a single run. Due the extremely large amount of data collected, the process is deeply dependent not only on storage ability but also on the bioinformatics capacity to produce valuable data and to evaluate the quality of the MPS platform (Kuiper 2016). Moreover, to compel an increasingly widespread application of MF the forensic community needs to be further educated: experts must address all the requirements of transparency (e.g., application

of legal standards for genetic privacy and reliable standards) and accuracy of the data produced (e.g., correct use of databases and informatics tools); while the end-users (i.e., crime scene investigators, lawyers, judges and juries) must be receptive to this new developments, acknowledging both its possibilities and limitation (Kuiper 2016; Clarke et al. 2017).

The present review on MF discusses how MPS techniques are already implemented as well as its potential applications to answer criminal and legal questions, organized in the topical issues: (i) determining cause of death; (ii) determining time of death; (iii) determining place of death; (iv) identifying suspects (Fig. 1).

Determining cause of death

In general, the presence of specific microorganisms can constitute evidence, helping to determine the cause of a given death, acting as bioindicators of criminal cases. In the following examples, we show some cases in which MPS was already implemented while in other instances, we discuss how such techniques could improve forensic analyses.

Drowning cases

During an autopsy, a common concern is the diagnosis of drowning, principally when the cadaver reaches an advanced state of decomposition due to an extended postmortem interval. The diatoms' presence in blood or organs (e.g., lungs, kidneys, spleen, liver and bone marrow) from the victims has been frequently considered as the gold standard indicator for drowning cases, discriminating between antemortem immersion (live entry) and postmortem submersion (dumping of a dead body) or even indicating the location of a probable drowning place (seawater vs. freshwater diatoms) (Sitthiwong et al. 2014; Spitz and Schneider 1964). However, this analysis presents several constraints: (i) diatoms may be present in low concentrations in some water sources, being difficult to detect even in some true drowning victims; (ii) some of these microorganisms present relatively large diameter/length (ranging between 2 and 500 μm), limiting their transposition of the alveolar wall and, consequently, their passage into the blood stream and organs; (iii) diatoms can be disclosed in several organs (e.g., kidney, liver and lungs) of non-drowned bodies (Calder 1984; Kakizaki et al. 2010; Kakizaki et al. 2009; Lee et al. 2017).

In the previous decade, attention has expanded to the use of other microorganisms — bacteria and cyanobacteria (Kakizaki et al. 2010; Lucci et al. 2008) and phytoplankton, including microalgae (Diaz-Palma et al. 2009) — using different approaches such as classical culture-dependent methods (Huys et al. 2012; Kakizaki et al. 2009; Lucci et al. 2008),

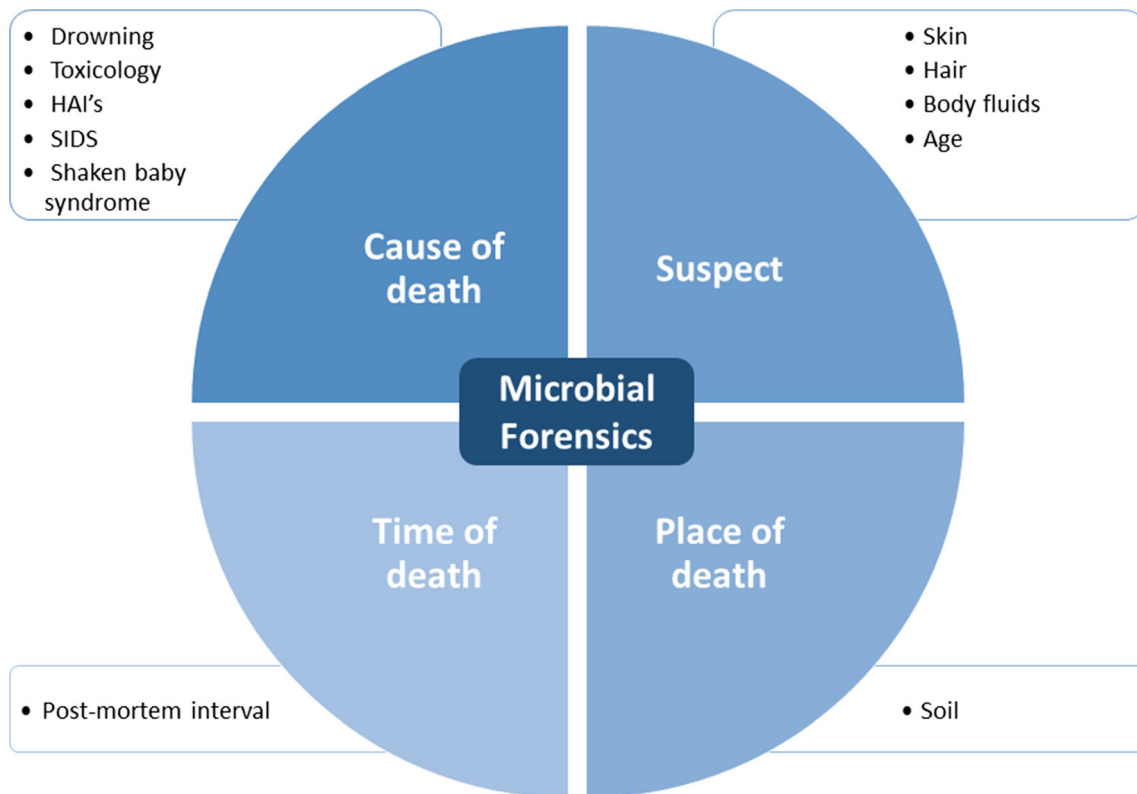


Fig. 1 Application of MF to answer criminal and legal questions

light (Sitthiwong et al. 2014) or electron microscopy (Lunetta et al. 1998) or PCR-based techniques (He et al. 2008; Huys et al. 2012; Rác et al. 2016; Tie et al. 2010). These other microorganisms are much more abundant and present smaller sizes (ranging between 0.2 and 2 μm) than diatoms and, therefore, at least theoretically, would provide more informative results. A recent study compared lungs, closed organs (kidney and liver) and cardiac blood between antemortem immersion and postmortem submersion, either using MPS or the 16S rRNA gene and culture-dependent identification of aquatic microorganisms using matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) (Lee and Eom 2016; Lee et al. 2017). The findings revealed that the microbial community in the closed organs was a significant marker for the diagnosis of death by drowning. Likewise, the same study hypothesized that drowning is associated with higher expression levels of surfactant protein A (regarded as a valuable marker of asphyxiation and acute lung injury) in the lung of true drowning victims (Lee et al. 2017).

Also, in the case of the presence of such microorganisms, through the comparison of the microbial communities present in the victim and the known environmental samples, it might be possible to reveal the place of death (or even discriminating between primary and secondary crime scenes). Additionally, comparing the microbiomes present in the victim and trace evidence found in the possible suspect(s), the data collected could be used to infer (or not) an association between them.

Toxicology

Although subjected to an intense debate, it has been hypothesized that during decomposition some microorganisms are responsible for the degradation of drugs (e.g., antidepressants, antipsychotics, benzodiazepines, cannabinoids, cocaine, heroin and morphine glucuronides) or poisons (e.g., cyanide) and for the neoformation of other metabolites (e.g., alcohol, methamphetamines and amphetamines, 4-hydroxybutanoic acid, methadone and other potent opioids) (Byard and Butzbach 2012; Gerostamoulos et al. 2012; Han et al. 2012). Therefore, postmortem microbial activity can, at least theoretically, interfere with autopsy findings concerning pre-mortem drug consumption and, consequently, alter the interpretation of the cause of death (Butzbach 2010; Butzbach et al. 2013; Castle et al. 2017; Drummer 2004; Gunn and Pitt 2012; Sastre et al. 2017; Skopp 2010).

In high-income countries, the consumption of antidepressant drugs is significantly increasing, with the collateral risks of producing addiction or increasing the risk of self-poisoning (Gjelsvik et al. 2014; Löfman et al. 2017). In MF, it is important to bear in mind that while some antidepressant drugs (e.g., amitriptyline, doxepin, fluoxetine, imipramine) present stable concentrations, others (e.g., dothiepin, phenelzine, tranylcypromine) are known to be degraded during cadaver putrefaction due to microbial activity (Butzbach 2010; Caddy and Stead 1978; Garcia-Pardo et al. 2018; Kwon and

Armbrust 2006; Monden et al. 2018; Pounder et al. 1994; Stevens 1984; Yonemitsu and Pounder 1993).

Benzodiazepines are frequently used to facilitate assaults and significantly increase the risk of road accidents (amongst others). Therefore, the accurate identification and quantification of these drugs are of primordial importance during autopsies. Several bacterial species, such as *Bacillus cereus*, *Staphylococcus epidermidis*, *Clostridium perfringens* and *Bacteroides fragilis*, are known to be responsible for bioconversion of nitrobenzodiazepines (e.g., clonazepam, diazepam, flunitrazepam, nimetazepam, nitrazepam, temazepam) into their 7-amino-metabolites under putrefying conditions, being the conversion rate dependent both on the tissue analyzed and storage conditions (Levine et al. 1983; Moriya and Hashimoto 2003; Robertson and Drummer 1998; Robertson and Drummer 1995; Stevens 1984).

The consumption of methamphetamines is rapidly increasing on a worldwide scale and, therefore, its forensic relevance. Several gastrointestinal microorganisms, such as species belonging to the genera *Enterobacterium*, *Enterococcus*, *Lactobacillus*, *Clostridium* and *Bacterioides*, are responsible for the *N*-demethylation of methamphetamines converting these compounds into amphetamines (Castle et al. 2017; Rommel et al. 2016).

The production of ethanol and other volatiles from a variety of substrates (namely carbohydrates, amino acids, glycerol and fatty acids) represents a common phenomenon amongst bacteria (more than 58 species; e.g., *Corynebacterium* sp., *Lactococcus garviae*, *Escherichia coli*, *Enterococcus faecalis*, *Klebsiella oxytoca*, *Klebsiella pneumoniae*, *Proteus mirabilis*), yeasts (more than 17 species; e.g., *Candida albicans*, *Candida tropicalis*, *Candida tropicana*, *Candida glabrata*, *Candida parapsilosis*, *Saccharomyces cerevisiae*) and fungi (more than 29 species; e.g., *Aspergillus awamori*, *Aspergillus foetidus*, *Aspergillus nidulans*, *Aspergillus oryzae*, *Aspergillus sojae*, *Aspergillus tamari*, *Fusarium* sp., *Rhizopus javanicus* and *Rhizopus oryzae*) (Boumba et al. 2012; Boumba et al. 2008; Skory et al. 1997). Therefore, the origin of postmortem ethanol can be attributed either to antemortem ingestion or postmortem microbial production (Boumba et al. 2013).

Gamma-hydroxybutyrate (GHB) has become a popular recreational drug, often consumed in combination with alcohol, being associated with several cases of drug-facilitated sexual assault. Therefore, during autopsy is frequently necessary to discriminate the cut-off concentration values between endogenous levels and exogenous exposure. It has been suggested a possible role of microorganisms in the postmortem production of GHB. Bacteria, such as *Pseudomonas* spp. (e.g., *P. aeruginosa*), present the enzymes necessary to convert gamma-aminobutyric acid (GABA; a main inhibitory neurotransmitter present in the central nervous system from mammals) to GHB (Elliott et al. 2004; Elliott 2004; Kalasinsky

et al. 2001; Kintz et al. 2004; Mazarr-Proo and Kerrigan 2005; Wells 2001).

The decrease in concentration of cyanide during the post-mortem period has been proven using blood and tissues (lungs, brain, liver and kidneys) of rabbits killed with potassium cyanide. This decrease is associated with the metabolism of cyanide into thiocyanates. Nevertheless, the bacteria involved in this process are still unknown (Ballantyne 2013; Ballantyne et al. 1974).

During an autopsy, the application of MPS-based technologies can provide a full screen of the microbiomes present in several body parts (e.g., blood, gut, brain). The occurrence of one or several of the previous referred microorganism delivers insights concerning possible postmortem variations in the concentration of these drugs and poisons (either by degradation, production or bioconversion) that may alter the results presented in the coroner report and misleading in the determination of the cause of death.

Hospital-acquired infections

Within the hospital environments, and despite all the imposed meticulous hygienization measures, there is circulation of bacteria (e.g., *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, *S. aureus*, *K. pneumoniae*, *Enterococcus faecium*, *Salmonella enterica* and *Clostridium difficile*), fungi (e.g., *Aspergillus ustus*, *Aspergillus flavus*, *Aspergillus fumigatus*, *Microsporium canis* and *Trichophyton tonsurans*), viruses [e.g., norovirus, respiratory syncytial virus (RSV), rotavirus, adenovirus, HBV and HCV] and other pathogenic agents (e.g., *C. albicans*), some of them multi-resistant to antibiotics that may trigger serious infections in ill-debilitated patients subjected to an intervention or undergoing immunosuppression treatments — hospital-acquired infections (HAIs) also known as nosocomial infections (NIs). Worldwide, the HAI rates are around 7% in developed and 10% in developing countries (Khan et al. 2017). In the United States of America are reported an annual a total of 1.7 million HAIs, 99,000 deaths and costs of 20 billion USD (Klevens et al. 2007; Sodhi et al. 2013). In Europe, the numbers are 3 million HAIs, 50,000 deaths and costs of 7 billion Euros (Bryers 2008). Besides this economic cost, HAI also create user insecurity and are a vulnerability hotspot of hospital environment and health system.

Over the precedent decade, HAIs have evolved continuously to a current state of significant growth. As a result, recent attention has been given by health governmental institutions (e.g., European Centre for Disease Prevention and Control and Centre for Disease Control) that have implemented specific surveillance programs to collect data on HAIs and have issued regulations for mandatory reporting of such infections (Herzig et al. 2015; Meier et al. 2008). Nowadays, pathogen monitoring in hospital surfaces is performed using conventional microbiology methods and luminescent

determination of ATP concentrations. However, these methods present several limitations and fail to reveal the true diversity of the microorganisms present, contributing to the significant worldwide rates of HAIs (Boyce et al. 2009; Shama and Malik 2013).

The main risk factors for the development of an HAI have been described as patient age, longer hospital stay duration, larger hospital size and the insertion of a central catheter. Also, several studies have indicated the potential routes of transmission of hospital-associated pathogens (HAPs) comprehend medical doctors' and nursing staff's clothing, stethoscopes, personal cellphones and computer keyboards (Lax and Gilbert 2015).

The use of robust and comprehensive techniques associated with MPS will allow comparing the microbiome present in hospital surfaces, patients, staff and visitors the bacteria responsible for the infection. The detection, identification and possible transmission routes of a given HAP are essential for the allocation of Infection Prevention and Control (IPC) resources and compelling constraint of infection. As well, in a case of a lawsuit, the genotyping data collected may be used to confirm or exonerate the hospital for the spread of the infection.

Sudden infant death and shaken baby syndromes

Sudden infant death syndrome (SIDS), the leading cause of post-neonatal infant mortality in developed countries, can be roughly defined as the unexpected death of a seemingly healthy infant in the postnatal period (i.e., the first year of life), remaining the cause of death unexplained after a meticulous investigation comprising autopsy, review of the clinical history and death scene investigation (Duncan and Byard 2018). The risk factors for SIDS have for long be regarded as multifactorial, resulting in the combination of factors such as the presence of an underlying vulnerability that increases susceptibility (e.g., genetics, undiagnosed pathology and immunologic disorders) and exposure to an exogenous stressor (e.g., sleeping position, exposure to tobacco smoke, alcohol and illicit drugs) (Duncan and Byard 2018). It has been reported that undiagnosed infections, attributed to several pathogens (e.g., viruses and bacteria), are responsible for 10% of the cases of SIDS (Morentin et al. 2012). The most common agents associated with SIDS are human herpes virus, Epstein Barr virus, cytomegalovirus, respiratory syncytial virus, adenovirus, human metapneumovirus, *Neisseria meningitidis*, *Haemophilus influenzae*, *Streptococcus pneumoniae* and *Bordetella pertussis* (Fernández-Rodríguez et al. 2006; Hawksworth and Wiltshire 2011). The viral infections are sometimes exacerbated by bacterial colonization and subsequent inflammatory response to endotoxins that cause hemorrhagic shock and encephalopathy. The unexpected death of children aged between 15 days and 1 year has been for long

a matter of several legal investigations, to exclude the possibility of a crime. Nowadays, routine postmortem microbiology protocols for SIDS comprise a limited number of viruses and bacteria (serology tests and PCR assays) performed in small quantities of specimens collected during autopsies. Efforts should be engaged to the development of comprehensive microbial studies providing etiologically specific disease diagnoses (Brooks et al. 2015; Fernández-Rodríguez et al. 2006; Krous and Byard 2005).

Shaken baby syndrome (SBS) is usually characterized by unexplainable intracranial, retinal hemorrhages and acute encephalopathy. The well-known Sally Clark case demonstrates the need of accurate microbiological analysis. The mother was sentenced for the homicide of both her children. However, the posterior evaluation of this case demonstrated a white blood cell profile compatible with a bacterial infection due to staphylococcal septicemia and meningitis. After spending several years imprisoned, the sentence was re-evaluated (Byard 2004a; Byard 2004b; Dyer 2005; Krous and Byard 2005).

As in the previous cases, if a comprehensive MPS-based panel (including both viruses and bacteria from several body locations — blood, cerebrospinal fluid, heart, lungs, spleen and throat — and body secretions — urine and feces) run during autopsy indicates the presence of such microbiological agents, it may constitute an effective tool to ascertain the causes of death. These microbial profiles should be accompanied with histological findings, positive C-reactive protein (CRP) measurement and antemortem clinical information.

Determining time of death

For law enforcement agents and forensic researchers, determining the time of death (postmortem interval — PMI) occupies a pivotal role in criminal investigations, mainly in suspicious death cases where no witnesses are present at the crime scene. An accurate estimation of the PMI may validate witness declarations, support the trustworthiness of supplementary physical evidence, provide reliable information for developing leads, simplify information management concerning decisions on investigative resources allocation and constitute evidence for later prosecutorial action (Maile et al. 2017).

Customarily a cornerstone of all death investigations, PMI can be estimated implementing two alternative approaches: (i) the assessment of antemortem changes, pathological or physiological, observed in the cadaver and combined with conclusions from investigations by the law enforcement agents (survival time), yielding rough estimations of the PMI (e.g., wound age estimation or evaluation of the gastric contents); and (ii) the assessment of postmortem changes evaluated at the crime scene, allowing investigators to conclude about the PMI, more accurate than the previously described methods

(e.g., body cooling, postmortem lividity, rigor mortis, supravital reactivity of skeletal muscle, vitreous potassium levels, autolysis, cerebrospinal fluid chemistry, H^3 -magnetic resonance spectroscopy, putrefaction and entomology). However, and despite all methods available, PMI can only be determined within certain probability limits (Madea 2015, 2016).

PMI estimation

For many years, forensic entomology, based on the postmortem succession of insects colonizing the cadaver, has been used for PMI estimation. In the recent years, evidence has been accumulated, proving that microbial communities can also go through successional changes postmortem, paving the ways for its use as PMI indicators (Dickson et al. 2011; Hauther et al. 2015; Heimesaat et al. 2012; Javan et al. 2016, b; Javan et al. 2017; Metcalf et al. 2013, 2016, b, 2017; Parkinson et al. 2009).

Microbiome approaches, based on the recent improvements both in sequencing platforms using phylogenetically and/or taxonomically informative gene markers (e.g., 16S rRNA, 18S rRNA, and internal transcribed spacer—ITS) and statistical and computational pipelines (e.g., machine learning), allow not merely to evaluate the microbial changes during PMI but also to analyze additional trace evidence signatures characterized by differences in microbial communities (Metcalf et al. 2017).

The postmortem microbiome—community of prokaryotic and eukaryotic microorganisms associated with the decomposition of animal carrion and human corpses—can be divided into two core components: the thanatomicrobiome and the epinecrotic microbial community. While the first is composed of microorganisms, mainly bacteria and fungi, present in the blood and internal organs (e.g., brain, heart, liver, lungs, spleen); the second is composed by the microorganisms residing in and/or moving on the surface (e.g., superficial epithelial tissues, oral mucosal membranes and distal orifices of the digestive tract) of decomposing cadavers. Contrary to the epinecrotic community, the thanatomicrobiome is more stable being unbiased by either abiotic (e.g., humidity, temperature and pH) or biotic factors (e.g., gasses, insects and scavenger activities) (Javan et al. 2016, b).

Upon death, because of the cessation of the activity of the immune system, the microorganisms present in the gastrointestinal tract are able to invade not only the surrounding tissues organs but also to move along the circulatory and lymphatic systems, initiating the decomposition process in a nutrient-rich environment, mediated by tissue autolysis (Carter 2017). In general, as decomposition progresses, microbial communities' diversity decreases with only a few genera becoming dominant. Additionally, a shift from an aerobic bacteria-based community (Phylum *Bacteroidetes*:

Bacteroides and *Parabacteroides*; Phylum *Firmicutes*: *Faecalibacterium*, *Phascolarctobacterium*, *Blautia* and *Lachnospiraceae incertae sedis*) to an anaerobic bacteria-based community (Phylum *Bacteroidetes*: *Clostridium*; Phylum *Firmicutes*: *Peptostreptococcus* and *Anaerospaera*; Phylum *Gammaproteobacteria*: *Wohlfahrtiimonas*, *Ignatzschineria*, *Acinetobacter* and *Providencia*) has been reported (DeBruyn and Hauther 2017). Therefore, it seems legitimate to infer that bacterial taxa present in internal organs are potentially useful for estimating the minimum PMI.

In a foreseeable future, the elaboration of a microbiome calendar produced with MPS-based technology, including samples collected at several time-points after death, will allow match the microorganisms present in a given cadaver and the time of death based on the proposed calendar.

Determining place of death

Another fundamental aspect in law enforcement and forensic researches is the distinction between primary and secondary crime scenes. While the first can be defined as the place where the criminal act was actually committed, the second is any other place (or places) related in a later time lapse, to this event (Lee et al. 2001).

As an example, the case of the massacre of civilians near Srebrenica (NE Bosnia) can be presented. In July of 1995, more than 8000 Bosnian Muslim males were executed and buried in a mass grave (primary crime scene) (Herman 2006). However, 3 months after the massacre, the cadavers were exhumed and transported to several other locations in Bosnia (secondary crime scenes) (Brown 2006). Although both scenes are critical for the crime reconstruction, the primary scene frequently contributes with more evidence to the forensic research. However, in several cases, only the secondary crime scene is available for the investigators. In this case, the identification of such mass graves was obtained using botanical and geological data. However, a similar approach, using microbial data, can be foreseen.

In forensic casework, soil analysis is frequently used as evidence, establishing a link between a suspect and an object, location or victim. This analysis studies intrinsic properties of soil such as mineralogy, chemistry, geophysics, texture and color. Soil, a complex matrix constituted by both organic and inorganic matter, although highly informative, is frequently overlooked when trying to conceal evidence. Furthermore, it adheres and persists on numerous surfaces (e.g., clothing, footwear, tires and tools) constituting a valuable trace material providing clues about the origin of an unknown sample, or to compare samples from a suspect with those from a crime scene. However, because of the significant similarity between samples collected at short distances, the substantial amounts of material required and the lack of robust soil databases, the

information provided by soil analysis has been quite restricted up to now (Young et al. 2014, 2015, 2017).

Soil microbiome

In the recent years, analysis of soil microbial communities has been regarded as potentially useful in forensics providing discriminatory power at fine scale resolution. Since the structure of the microbial community is deeply determined by several factors (e.g., soil type, seasonal variation, site management, vegetation cover and environmental conditions), bacterial and fungal communities possess an extremely site-specific profile (Grantham et al. 2015). The recent advances in MPS can generate thousands of sequencing reads per sample, providing a much more comprehensive snapshot of microbial communities present in a given soil sample (Demanèche et al. 2017; Young et al. 2014, b, 2015, 2017). Depending on the type of soil, a single gram may comprehend up to 8.3 million unique sequences (Roesch et al. 2007).

Recent studies have demonstrated that during the decomposition process, the surface and buried soil communities presented diverse behaviors. While microbial communities from surface soil present a decreasing trends in taxon richness, diversity and evenness; microbial communities in close contact with buried cadavers present the opposite characteristics (i.e., increasing taxon richness, consistent diversity and decreasing evenness). Also, *Proteobacteria* has been referred as the most abundant phylum in grave soil samples, the relative abundance *Acidobacteria* decreased and *Firmicutes* increased in surface cadaver-soil communities, while the microbial community composition remained rather constant in buried soil communities (Finley et al. 2015, 2016).

Given the complexity of soil microbial communities, the application of MPS will significantly accelerate the identification of such communities. Although a daunting endeavor, it would be rewarding to construct comprehensive maps of the microbial communities' geographic distribution, such as the works recently publish by Thompson and co-workers (2017) and Delgado-Baquerizo and co-workers (Delgado-Baquerizo et al. 2018); these databases would certainly be useful to identify the place of death at least in broad areas scales.

Identifying suspects

A recent study revisited the ratio of bacterial/human (B/H) cells, estimating that in a reference man (age 20–30 years; weight 70 kg, height 170 cm), there are 3.8×10^{13} bacterial and 3.0×10^{13} human cells, corresponding to a B/H = 1.3 (uncertainty of 25%, variation of 53%) and a total mass is about 0.2 kg (Sender et al. 2016a, b). This values differ from the previously proposed 10:1 ratio (Savage 1977). In those earlier studies, only nucleated human cells (0.3×10^{13}) were

considered, disregarding the population of non-nucleated red blood cells (Sender et al. 2016b).

The human microbiome should not be regarded as an indivisible whole but as a sum of parts. Several lines of evidence point out towards this direction: (i) these bacteria are unevenly distributed through the different anatomical sites (e.g., colon — 10^{14} ; dental plaque — 10^{12} ; ileum — 10^{11} ; saliva — 10^{11} ; skin — 10^{11} ; stomach — 10^7 ; duodenum and jejunum — 10^7); (ii) each anatomical site/location presents a specific taxonomic composition; (iii) these microbiomes present seasonal patterns influenced by several factors (e.g., environmental exposure, age, dietary preference and antibiotic or antiseptics use) (Cho and Blaser 2012; Ding and Schloss 2014; Lloyd-Price et al. 2017; Sender et al. 2016b). However, to be of any use in human identification, individual microbiome stability and differentiation must be evaluated, a task far from being easily performed and likely unreachable (Arumugam et al. 2011; Franzosa et al. 2015; Lax et al. 2015).

Beyond individual identity, the human microbiome research may also disclose unpredicted information. Therefore, and despite being highly regulated, human microbiome research still poses several ethical, legal and social challenges. Microbiomes may provide information on people origins or ethnic background and past exposures or countries/geographical locations previously visited. This intelligence may be used to highlight the subject as a person of interest for homeland security and law enforcement services; breaching individual's right to privacy. Moreover, human microbiotas are associated with both health and homeostasis. The occurrence of certain microorganisms may reveal individual's susceptibility and predispositions to certain clinical conditions such as obesity or diabetes (correlation between intestinal microbiota) and local or systemic infections (colonization of the nasopharynxes by *S. aureus*), contributing for socioeconomic discrimination or stigmatization. Ultimately, microbial sample collection (e.g., collection of stool samples and vaginal swabs) can be regarded as invasive and culturally non-acceptable amongst certain sub-sectors of the society (McGuire et al. 2008; Hawkins and O'Doherty 2011).

Skin microbiome

The human skin surface is colonized by a substantial number of microorganisms that can be easily dispersed and transferred to surfaces upon touching (Fierer et al. 2010). The human microbiome can be used as a personal signature, establishing a link between individuals and the objects they touch, such as cell phones and computers (keyboards, touchpads and other devices' surfaces) (Fierer et al. 2010; Lax et al. 2015; Meadow et al. 2014). The degree of diversity of the skin microbiome depends on the depth of the study, if only the phylum of the bacteria is considered, only a few distinctive taxa can be identified amongst various persons. However, increasing the

taxonomic depth, if identification is achieved at the genus, species and strain population levels, the diversity is extremely specific for each person, acting as an individualized signature, with a discriminatory power similar to a fingerprint (Blaser 2010; Costello et al. 2009; Fierer et al. 2008; Gao et al. 2007; Grice et al. 2009). Additionally, each body part constitutes a specific niche, with a particular microbiome composition, dictated by several factors (e.g., skin thickness and folds, densities of hair follicles and the presence of both eccrine and apocrine glands). For instance, if *Propionibacteria* and *Staphylococcus* species dominate sebaceous sites and *Corynebacteria* moist sites, β -Proteobacteria and Flavobacteriales dominate dry sites. However, these differences remain relatively stable throughout time and despite of the personal hygiene. Additionally, the bacteria constituting this microbiome persist on touched surfaces for extended periods since some of these microorganisms are extremely resistant to environmental stresses (moisture, temperature and UV radiation) (Brooke et al. 2009; Kong and Segre 2012; Smith et al. 1996). It remains, however, in doubt the stability of the personal profile and changes induced by antifungal and antibiotic therapies (Langdon et al. 2016; Raymond et al. 2016).

Hair microbiome

Hairs are amongst of the most commonly found evidence collected at crime scenes. However, the vast majority lacks the anagenic root (actively growing hair), essential for DNA extraction and posterior analysis employing conventional typing methods, such as STR profiles (Tridico et al. 2014; Tridico et al. 2017).

A recent study compares the microbiome present both in from human scalp and pubic hairs and discuss the use of these traces in criminal and civil investigations (Tridico et al. 2014). The majority of hairs recovered in forensic investigations correspond to scalp hairs while pubic hairs are used in sexual assault cases. According to the authors, data collected from the microbiome analysis allowed not only the individualization of the contributor but also to discriminate between the two sources of hair. The aforementioned study also revealed that cohabiting and sexually active couples interchange microbiomes during sexual intercourse. Additionally, the microbiome present on scalp hairs has been shown as more prone to compositional oscillations than pubic hairs (Tridico et al. 2014).

Body fluid microbiome

At crime scenes, the most commonly found body fluids are blood, semen and saliva. However, other fluids (e.g., vaginal fluid, urine and sweat) can also be present and perform meaningful roles in investigations, namely as DNA evidence (Virkler and Lednev 2009). It has been proposed that each

body fluid type presents a specific composition of microorganisms that can be used as bioindicators, being possible to infer the type of body fluid present based on its microbial composition (Hanssen et al. 2017). For instance, saliva could be identified by detection of specific bacteria, such as *Veillonella atypica*, *Streptococcus mutans* and *Streptococcus salivarius*; while vaginal fluid could be identified by detection of specific bacteria, such as *Lactobacillus crispatus* and *Lactobacillus gasseri* (Choi et al. 2014; Giampaoli et al. 2017).

When considering lower taxonomic levels (i.e., genus, species and strains), the human microbiome is utterly characteristic from a given individual (Blaser 2010). Therefore, the fine resolution of the results acquired from MPS-based techniques will allow to identify suspects comparing the microbiomes or, at least theoretically, establish an association between perpetrators and victims.

Conclusions

Over the preceding decade, the advances in MPS and related techniques have revolutionized science in many numerous fields, from individualized medicine to forensics. Nowadays is possible to sequence complete genomes or complex samples in a few hours at an affordable price. As such, these innovative techniques can be used as a complement to classical microbiological methods in a polyphasic approach (Tomaso and Neubauer 2011).

As previously suggested (Amorim 2010), one of the most prominent breakthroughs associated with microbial forensics might be reached in the cases of sexual offenses, associating offender and victim even several years after the crime has been committed and using as bioindicators sexual transmitted diseases (e.g., HIV and HCV). This possibility was proven in a case in which a step-father was convicted of abusing his 10-year-old stepdaughter, based on the phylogenetic analysis of the HIV transmitted (Banaschak et al. 2000).

However, as previously referred, prior to its wide implementation in forensic investigations, several steps should be considered (Budowle et al. 2014, 2017). Indeed, it is necessary to define standard operational protocols for sample collection, handling and preservation (Budowle et al. 2006). All methods should be further validated, concerning aspects such as sensitivity, specificity, reproducibility, repeatability and limit of detection (Budowle et al. 2014). Finally, the creation of reliable and comprehensive databases is mandatory for the accurate interpretation of the obtained results (Skopp 2010).

Author contribution Drafting and critical revision of the manuscript: MO, AA.

Funding This study was funded by FEDER - *Fundo Europeu de Desenvolvimento Regional* funds through the COMPETE 2020 - Operacional Programme for Competitiveness and Internationalisation (POCI), Portugal 2020, and by Portuguese funds through FCT - *Fundação para a Ciência e a Tecnologia/Ministério da Ciência, Tecnologia e Inovação* in the framework of the project “Institute for Research and Innovation in Health Sciences” (POCI-01-0145-FEDER-007274).

Compliance with ethical standards

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

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors.

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