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Review Paper

Microbes as forensic indicators

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Abstract. The forensic potential of microorganisms is becoming increasingly apparent as a consequence of advances in molecular sciences and genomics. This review discusses instances in which microbes, and in particular bacteria, can impact upon forensic investigations. There is increasing evidence that humans have an extremely diverse 'microbiome' that may prove useful in determining ethnicity, country of origin, and even personal identity. The human microbiome differs between regions of the body and may prove useful for determining the nature of stains such as those caused by saliva and vaginal fluid: it may even be possible to link the stains to the person responsible for them. Similarly, the composition of the microbiome present in a soil sample may prove a useful indicator of geographic origin or as a means of linking people, animals, or objects together or to a specific location. Microorganisms are important in the decay process and also influence the presence and concentration of alcohol, drugs, and other chemicals of forensic relevance. There is also a possibility that the entry of microorganisms into the body during the agonal period may prove useful for the diagnosis of drowning. The transmission of infectious diseases, and in particular sexually-transmitted diseases, can provide evidence linking a victim and a suspect. Microorganisms that cause fatal infections are not always identified at the time of death and may lead to the death being considered 'suspicious'. If a fatal infection can be linked to a hospital or medical procedure it can lead to prosecutions and therefore it is important to determine when and where an infection was acquired. Similarly, naturally acquired infections need to be distinguished from those that result from malicious transmission. Microorganisms can therefore provide evidence in many different forensic scenarios but most of the work is still at the experimental stage and there are therefore many opportunities for further research.

INTRODUCTION

The group of microorganisms (or "microbes") is a diverse collection of organisms linked by their size. Microbes comprise: bacteria, which are prokaryotes; fungi, algae, parasites and some helminths, which are eukaryotes; and viruses and prions, which are termed akaryotic. Organisms which are identified as 'bacteria' are classified in two phylogenetic domains, namely Bacteria and Archaea. Interestingly, the latter are in fact more closely related to the Eukarya.

Although the Archaea are often associated with extreme environments such as deep sea vents, they are also an often overlooked part of the human microbiome (Dridi *et al*., 2011). Microorganisms are abundant and ubiquitous in all terrestrial and aquatic environments. For example, it is commonly stated that there are over ten times more bacteria living within and upon the human body than

there are human cells (Turnbaugh *et al.*, 2009). Microbes have long been recognised as important in fields such as medicine, ecology, and fermentation science but they have been largely ignored by forensic scientists. However, this is likely to change as rapid advances in molecular sequencing and computational techniques are bringing about a revolution in the way we approach the study of these organisms (Li *et al.*, 2012; Segata *et al.*, 2012). For example, it is no longer necessary to culture the organisms in order to identify them and the new science of metagenomics enables characterisation of the hundreds or thousands of microorganisms that constitute the microbial community, or 'microbiome', of an ecosystem (Human Microbiome Project Consortium 2012a, b; Yatsunenko *et al.*, 2012). Similarly, it is now possible to study the transmission chains of particular bacterial strains and plasmids more effectively (MacConaill & Meyersen, 2008; Nakamura *et al.*, 2008; Pallen & Loman, 2011). The omnipresence and diversity of microbes means that they are a potential source of forensic evidence. Indeed, the term 'microbial forensics' is now applied to the study of how micro-organisms can contribute to forensic investigations (Breeze *et al.*, 2005). For example, the presence of a particular microbial community could be used to characterise an individual, organism, object, or location. In addition, some micro-organisms are pathogenic and whilst most infectious diseases are readily diagnosed, they may contribute to some contentious cases in which a person has died from no obvious cause. Microbes have also been spread recklessly or maliciously and it is therefore necessary to link the accused and the plaintiff to a specific strain. In addition, the diversity of microbial metabolism means that most organic materials and many inorganic substances can be altered by their activities and this may affect the preservation of other forms of forensic evidence. Finally, of course, a dead body can be a source of infectious micro-organisms and should always be handled carefully to avoid the risks of transmission (Malik & Singh, 2010).

Role of microbes in decomposition processes

Microbes are seldom present in the blood stream or cerebrospinal fluid of a healthy individual, but when a person dies micro-organisms such as bacteria and fungi living on the skin and within the gut can proliferate throughout the body as physical and immunological barriers start to break down. However, there is little evidence to support the conjecture that microbes enter the body during the agonal period to any great extent – that is during the period during which death takes place (Morris *et al.*, 2006). From a medico-legalistic perspective, the agonal period is difficult to define and one suggestion is that it is the time between the cardio-respiratory system ceasing and the brain no longer functioning (Mason, 2010). After death, the speed with which microbes move through the body is affected by the environmental conditions and whether or not the dead person suffered wounds that facilitated their entry. If the body is frozen immediately after death, then, obviously, there is no movement of microorganisms and decomposition does not take place. Morgue refrigerators typically operate at -1°C to +4°C and therefore delay but do not prevent the decay process. However, provided the body is placed in cold storage shortly after death, this is usually sufficient to reduce the likelihood of microbes invading the body for at least 24 hours (Morris *et al.*, 2007a). This is an important consideration when there are concerns about whether organisms recovered at post-mortem contributed to the person's death.

As the environmental temperature increases, the microorganisms present upon and within a dead body will replicate faster and as a consequence the pH of the blood becomes acidic and the fluids and tissues become anaerobic. High humidity can also facilitate the growth of micro-organisms and therefore decay is faster in the humid tropics than it is in cool

temperate climates. The Gram-positive bacillus *Clostridium perfringens* can come to dominate the microbial community since it has a doubling-time of only 8 minutes under optimal conditions (Spicer, 2000) – which is faster than most other bacteria typically found on a dead body. Bacterial putrefaction results in the loss of body tissues (and hence, of potential evidence) and generates gases such as carbon dioxide, hydrogen sulphide, and methane, that are responsible for the bloat stage of decomposition. In addition, phenethylamine, tryptamine and other amines are generated (Stevens, 1984). Bloat often ruptures the skin surface and this allows the entrance of oxygen thereby facilitating the return of aerobic decay processes. Volatile chemicals such as mercaptans released by bacteria attract invertebrate and vertebrate detritivores that then feed on the corpse and contribute to the loss of tissues. To date, relatively few studies have been done on the microbiome of dead bodies in a forensic context. Consequently, although it is to be expected that the microbial species profile will change as the body undergoes the various stages of decay it is not yet possible to use this as an indicator of the time since death. Similarly, a dead body can be expected to alter the microbial abundance and profile of the soil or other matrix on which it is found in a time-dependent manner but there is as yet limited information on this (Howard *et al.*, 2010).

In the process of 'wet drowning', the victim breathes in fluid that damages the lining of the lungs; in freshwater drowning, large volumes of water rapidly pass across into the circulation. Microscopic particles suspended in the fluid can pass across the surface of the alveoli and, as long as the heart is beating, become swept around the body. Drowning is a notoriously difficult diagnosis to the make on the basis of pathology (Saukko & Knight, 2004) but the recovery of diatoms from the organs and bone marrow is recognised as a good indicator (Hardy & Wallace, 2012). Diatoms are single-celled algae that form silicaceous cases called 'frustules' that are resistant to decay and aid in their identification. The abundance and diversity of diatoms varies between ecosystems and times of the year (Compton, 2011) and can therefore provide an indication of where and when a person drowned (Pollanen, 1997, 1998, Pollanen *et al.*, 1997). However, using diatoms as a marker of drowning does have some limitations. The extraction of diatoms is not easy and they are not present in all water sources or may not be abundant. Their comparatively large size (2-200µm) can restrict their movement into the circulation. The inability to recover diatoms therefore cannot be taken as an indication that the victim did not die of drowning. There is also a lack of consensus on the extent to which diatoms enter the circulation during day-to-day life via the diet or being breathed in. Some workers have therefore suggested that the recovery of bacteria from the blood could provide an alternative means of diagnosis. Microbial diversity also varies between habitats but they are much smaller (typically 0.5-5µm) than diatoms and much more abundant – and therefore more likely to be recovered. For example, Kakizaki and his co-workers have demonstrated that marine species of *Vibrio* bacteria which are bioluminescent can be isolated from the blood of victims of drowning (Kakizaki *et al.*, 2009; 2011 a,b). Similarly, because many marine and freshwater water sources are contaminated with human and animal sewage, it has been suggested that the presence of faecal coliform bacteria and faecal streptococci in the blood could also indicate drowning (Lucci *et al.*, 2008; Suto *et al.*, 2009). However, to use the identification of any of these bacteria in support of a case that a person had drowned, it would have to be indisputable that there were no alternative routes by which the organisms could have entered the body. The victim should therefore not have suffered any wounds at the time of their death and the body would have to be recovered before the decay process was advanced.

Microbes and post-mortem toxicology

During the decay process, microbes degrade certain drugs and also generate metabolites that can be mistaken as indicators of pre-mortem drug consumption. For example, nitrobenzodiazepines such as Clonazepam and Nitrazepam, are quickly broken down by bacteria to amino compounds and may be difficult to find in the blood even when the victim has died of an overdose (Robertson & Drummer, 1995; Drummer, 2007). By contrast, morphine can be detected in buried bodies up to 8 years after death (Skopp, 2004; 2010) although during this time morphine-3-glucuronide is converted to free morphine by bacteria (Carroll *et al.*, 2000). In a notorious case in the UK, it was the resistance of morphine to autolytic and putrefactive processes that provided evidence that Dr Harold Shipman was responsible for intentionally killing up to 240 of his patients by administering overdoses of diamorphine (Pounder, 2003). If he had used a more labile drug it is possible he may never have been convicted.

Bacterial and fungal fermentation produces a range of short chain alcohols, although not methanol, from a range of metabolites. This needs to be considered in cases in which alcohol consumption might have contributed to the cause of death (for example, intoxication, car accidents or aeroplane crashes). The amounts and rates of ethanol produced vary between species of microorganism and substrate (Corry, 1978) but can be enhanced by the presence of high blood sugar levels at the time of death. For example, Appenzeller *et al.* (2008) describe a case in which exceptionally high levels of alcohol were found in the blood of a 14-month old child at autopsy. This led to a suspicion that the child may have been a victim of abuse (given the alcohol) or neglect (allowed access to the alcohol). It was subsequently demonstrated that the child had been given a glucose infusion shortly before her death and the alcohol detected was a consequence of fermentation by *Lactococcus garvieae*. This bacterium is seldom recovered from humans and is unlikely to have been the cause of death (which was not stated in Appenzeller's paper) and it is possible that it gained access to the body iatrogenically (for example via a contaminated intravenous drip).

Microbial ethanol production is also affected by environmental factors such as the temperature, humidity, and oxygen levels, although it usually takes at least 3 days for appreciable levels to develop (Leikin & Watson, 2003). According to some authors, the presence of 1-propanol (n-propanol) in blood or tissues is a good indicator of bacterial fermentation, because it is not typically present in alcoholic drinks. However, Skopp (2004) does not consider this to be especially reliable because 1-propanol is formed at variable rates and therefore cannot be used as an indicator of the extent to which bacterial fermentation has occurred. These products of microbial metabolism may form *de novo* within the blood or diffuse into it from body cavities, such as the gut or the lungs. For example, Furumiya *et al.* (2012) describe an unusual case in which a man was suffocated under a pile of wood chips, some of which he inhaled. Over the following 24 hours, the wood chips in his lungs were fermented by bacteria and resulted in alcohol being recovered from his blood. The amounts of ethanol produced in this way post-mortem are generally small (<0.07% [0.660 mg/g]) but instances of levels of up to 220 mg/dL [2.075 mg/g]) have been reported (Zumwalt *et al.*, 1982; O'Neil & Poklis, 1996). For this reason, if fermentation is thought to have occurred it is advisable to measure the ethanol concentration of fluid extracted from the vitreous humor where there is less likely to be bacterial growth.

Microbes have also been linked to both the production and break down of gamma hydroxy butyric acid (GHB). GHB is of forensic importance because although it is naturally present at very low (nano-molar) concentrations in the body, it is also used voluntarily as a recreational drug and maliciously as a sedative in cases of 'date rape'. The discovery of high GHB

concentrations at post-mortem could therefore indicate either an overdose or involvement in a sexual assault. GHB is produced by natural decay processes and also by a variety of bacterial species, such as *Pseudomonas aeruginosa* (Elliott *et al.*, 2004; Nishimura *et al.*, 2009). The interpretation of GHB levels in post-mortem samples is therefore problematic (Elliott, 2004) and collection of post-mortem blood into tubes containing sodium fluoride as a preservative is recommended to restrict microbial growth (Beránková *et al.*, 2006). It is not yet possible to relate GHB levels to the microbiome composition or abundance.

Microbes as a means of identifying individuals

People or bodies are usually identified from their DNA and morphological characteristics. In the case of trace evidence, such as body fluids, human DNA is also the best identifier, whilst fingerprints and other contact evidence are analysed using their physical characteristics. However, it is not always possible to extract a full DNA profile and fingerprints are often smudged and incomplete. Studies on the human microbiome indicate that not only are there major differences in the microbial composition within regions of the body but that there appear to be consistent differences between individuals (Costello *et al.*, 2009). This has led to suggestions that people may have unique microbiomes and these could be used as a means of identification (Blaser 2010). For example, Fierer *et al.* (2010) demonstrated that it was possible to link people to the computer they had used by analysing the skin bacteria they had left behind on the keypad. However, Tims *et al.* (2010) had less success in their attempts at using skin microflora as a forensic indicator of country of residence and clearly more studies are needed in this area.

Much of the work to date has been undertaken on the forensic potential of the salivary microbiome. This is because human bite injuries are often inflicted by both assailant and victim during the fatal and non-fatal assaults and therefore form an important source of forensic evidence linking the two together. Unfortunately, although it is generally accepted that all humans have a unique dental arrangement, their bite marks are often difficult to interpret (Sweet & Pretty, 2001). Furthermore, although DNA is transferred during biting, it is degraded by enzymes present in the saliva (Yaegaki *et al.*, 1982; Sweet *et al.*, 1997) and therefore it becomes increasingly difficult to recover a full DNA profile with the passage of time. Saliva has been estimated to contain 700 diverse bacterial species at a density of 1.4 x 10^8 organisms ml^{-1} (Lazarevic *et al.*, 2011). The composition of the salivary microbiome is affected by oral hygiene, diet, geography, and genetics but more work is needed to confirm whether it can provide a consistent and reliable indicator of individual identity (Lazarevic *et al.*, 2010). Viable bacteria can be recovered from bite sites on skin and clothing for at least 24 hours after being inflicted (Brown *et al.*, 1984; Borgula *et al.*, 2003; Rahimi *et al.*, 2005) but since many of the species cannot be reliably cultured *in vitro*, the use of salivary microbes as forensic indicators will depend upon techniques such as high throughput screening (Ahn *et al.*, 2011; Lazarevic *et al.*, 2011).

Microbes as a cause of death

As a general rule, the recovery of a single microbial species from body fluids at autopsy suggests that infection occurred during life, whilst mixed species profiles indicate post-mortem invasion. However, this is not invariably the case and it can be difficult to avoid contamination when taking the samples (Tsokos & Püschel, 2001; Morris *et al.*, 2006). Indeed, in a well-publicised case in the UK, a pathologist giving evidence to an inquiry on the death of an 8-week-old child failed to report recovering *Staphylococcus aureus*, from the cerebrospinal fluid and other regions of the body because this is an extremely common component of normal skin flora and he was sure that it was a contaminant.

There was no immediately obvious cause of the child's death but as this was the mother's second child to die in infancy, suspicions were aroused. The case went to trial and the mother was found guilty of killing both her children, the first by smothering and the second by shaking him (shaken baby syndrome) and sentenced to life in prison. The microbial evidence subsequently came to light, along with the child's white blood cell profile suggesting a bacterial infection. This therefore indicated that staphylococcal septicaemia and meningitis may have caused the child's death. The mother was released after spending several years in jail and her conviction declared 'unsafe' (Byard, 2004; Dyer, 2005).

The sudden death of otherwise apparently healthy children during infancy invariably causes consternation and accusations. In some cases, death is ascribed to 'Sudden Infant Death Syndrome' (SIDS) but there is little agreement on its causes (Goldwater, 2011). One possibility is that it can be precipitated by a transient bacterial infection which, although cleared before death occurs, generates toxins that cause haemorrhagic shock and encephalopathy (Morris *et al.*, 2007b; Weber *et al.*, 2008). Interestingly, this can result in pathology that is also associated with so-called 'shaken baby syndrome'. It is therefore important to test for immunological evidence of response to bacterial toxins in samples because even if it is not possible to isolate organisms in the blood or cerebrospinal fluid, this would provide evidence of a recent bacterial infection.

Nosocomial transmission of microbes

Nosocomial transmission (also referred to as 'Healthcare Associated Transmission' and 'Hospital Acquired Infections') is where a disease is transmitted within a medical setting such as a hospital or as a consequence of a medical procedure such as an injection or insertion of a urinary catheter. Hospitals and medical centres are, inevitably, full of sick people and medical procedures often involve puncturing the body and/or inserting implements. The opportunities for disease transmission are therefore high and while it is impossible to prevent them entirely, there are usually clear guidelines and protocols in place. When a patient acquires an infection through these not being followed, it is possible that the consequences for the patient may be severe or even fatal. In such a situation a claim for medical negligence could be made and in some cases there may be grounds for a criminal prosecution. It can therefore be important to demonstrate whether a patient acquired their infection before they were treated and whether an infection could be linked to a specific member of medical staff or medical equipment. For example, in USA, several cases of bacterial meningitis caused by the oral bacterium *Streptococcus salivarius* were linked to an anaesthetist who had administered spinal injections whilst not wearing a facemask (Shewmaker *et al.*, 2010; Srinivasan *et al.*, 2012).

Natural transmission of microbes

The natural transmission of microbial pathogens is normally a concern for the health authorities. However, when pathogens that are associated with bioterrorism (see later) are recovered it can result in police involvement. For example, anthrax has caused the deaths of several heroin users in the UK and elsewhere in Europe (Christie, 2010; Knox *et al.*, 2011). The risks appear to be greatest for those injecting the drug rather than smoking it (Palmateer *et al.*, 2012). Most of the heroin consumed in the UK and Europe is sourced from Afghanistan where *Bacillus anthracis* is endemic. However, it has not yet been proven that the anthrax also originates from Afghanistan or how it might be contaminating the heroin. Similarly, there are sporadic cases of people in the UK and USA contracting anthrax from untreated animal hides used to make traditional drums, such as djembe drums (Stratidis *et al.*, 2008). Some of the affected people died from inhalation of anthrax which is extremely unusual in

cases of natural human infections (although it can be a feature of the disease when it is used as a biological weapon). Marston *et al.* (2011) used Multiple Loci Variable number of tandem repeats Analysis (MLVA) and canonical Single Nucleotide Polymorphism (canSNP) analysis to investigate five of the American cases of anthrax in individuals handling animal hides. In one of these, the anthrax was probably derived from Pakistan whilst two cases in Connecticut and one in New York were shown to have been infected with the same genotype of *B. anthracis* (MLVA-8 GT 1), which was thought to have originated from West Africa. Lack of information on the epidemiology of anthrax genotypes in Africa made it impossible to be more precise. The final case occurred in New Hampshire and had a new MLVA-8 genotype (GT 149) and as no information other information was available on the source of the drums it was not possible to determine the geographical origin of the anthrax. This study demonstrates the effectiveness of genotyping in linking infections and determining the source of the disease.

Naturally acquired infections, especially those with a restricted distribution, are a potentially useful forensic indicator of geographical origin. For example, wild animals and plants often harbour parasites and diseases that are absent from those that are captive bred owing to treatment or the absence of a suitable vector. This might prove useful in cases of alleged wildlife crime in which there is a dispute over the origin of an animal. Indeed, the illegal trade is recognised as a potential source of emerging diseases (Rosen & Smith, 2010).

Sexually transmitted microbial diseases

Sexually transmitted bacterial infections such as *Neisseria gonorrhoeae* and *Chlamydia trachomatis* are highly prevalent in many parts of the world (World Health Organization, 2011) and there are concerns over the spread of multi-drug resistant strains (Wang *et al.*, 2005; Deguchi *et al.*, 2010). The transmission of these infections between willing sexual partners above the age of consent seldom results in criminal proceedings but civil actions may result (see 'reckless transmission'). Where the infection was acquired as a consequence of sexual assault, genotyping of the bacteria from the plaintiff and the defendant could potentially be used to link the two (or more) people together (Lowe *et al.*, 2009). This might be useful in cases in which semen samples were not taken at the time of the alleged assault or the human DNA evidence was not incriminating. For example, the case might not be reported to the police until months after it took place and recovering the DNA of a man from the bed sheets of a female family member cannot be considered suspicious and certainly does not indicate that he had sex with her. At present, there are concerted efforts to identify genetic markers to study the epidemiology of *N. gonorrhoeae* (Unemo & Dillon, 2011) and these could prove useful in a forensic context (Black *et al.*, 2009). However, unless the strain involved was particularly rare, it would be difficult to establish strong proof of association between two adults. In the absence of medical records it would also be difficult to establish the direction of transmission. This is because *N. gonorrhoeae* is a common infection among sexually promiscuous adults (or those who have a sexually promiscuous partner) and therefore if two adults share the same locally circulating strain of *N. gonorrhoea* it is only weak circumstantial evidence of transmission from one to the other. However, this would be different in cases of gonorrhoea in children below the age of sexual consent (Hammerschlag & Guillén, 2010), since *N. gonorrhoeae* is transmitted almost exclusively through sexual intercourse. Therefore, a child generally only acquires the infection through a criminal act having taken place. Martin *et al.* (2007) describe a case in the UK in which molecular genotyping of *N. gonorrhoeae* was used to help convict a man of sexually abusing a young female relative. The abuse became apparent when

the child was diagnosed with the infection and the same rare sequence type was identified from stained regions of the suspect's underpants. The acceptability and strength of the evidence was never tested in court because the accused confessed when presented with it before the trial. Perinatal transmission of *N. gonorrhoeae* can occur when the mother has an active infection, but is usually manifested as an ophthalmic infection in babies rather than in the genitalia (Woods, 2005). According to Goodyear-Smith (2007) it is also important to consider the possibility of contaminative transmission of this organism, because under suitable conditions, the bacterium can remain infective on discharges, contaminating hands, communal towels, and clothing for several hours or even days. This is thought to explain instances of *N. gonorrhoeae* infections spreading rapidly among pre-pubertal girls living in children's institutions reported during the late C19 and early C20 (Goodyear-Smith, 2007). Ahmed *et al.* (1992) described an 'epidemic' of gonorrhoea among young girls living in a Somali orphanage which they ascribed to transmission during sexual abuse by a male guardian. Certainly, the serotyping evidence (genotyping was not available) was incriminating, but it is possible that after some of the girls were sexually abused the disease subsequently spread by contamination. *Chlamydia trachomatis* can also be acquired perinatally and owing to the high natural prevalence of infection among women it has been estimated that up to 100,000 neonates are exposed to infection annually in the USA alone (Darville, 2005). Most neonatal infections give rise to ophthalmic disease or pneumonia and they have also been implicated in the development of SIDS (Lundemose *et al.*, 1990). Occasionally, neonatal infections can persist for several years in a child's vagina and anus (de Barbeyrac *et al.*, 2010) but these are unusual and infections at these sites in children are generally considered to be an indication of abuse (Black, 1997).

'Reckless transmission' of microbial diseases

The fear generated by the Acquired Immune Deficiency Syndrome (AIDS) epidemic stimulated many countries to introduce legislation that makes it a criminal offence for a person who knows that he/she is carrying Human Immunodeficiency Virus (HIV) to fail to inform a sexual partner of their condition and then transmit the infection to them through unprotected sex. This is often referred to as 'reckless transmission' and can be punishable by imprisonment (Weait, 2005; Bruce-Chwatt, 2012). This has proved highly controversial because some commentators have argued that this will make people less likely to undergo voluntary testing for the disease: if one does not know for certain that one is HIV-positive then one cannot be accused of knowingly transmitting it (Lowbury & Kinghorn, 2006). HIV, like other RNA viruses, exhibits a rapid rate of mutation and it is therefore not possible to match exactly the viral profile of alleged donor and recipient. Neither is it possible to demonstrate the direction of transmission or provide conclusive proof that other parties were not also involved in the transmission chain (Learn & Mullins, 2004). Consequently, analysis of viral profiles can only indicate that the two profiles are statistically extremely similar. For a successful prosecution, further evidence would be required.

It is probable that criminal prosecutions for passing on other sexually-transmitted diseases will increase once technology becomes available that can establish a reliable link between the plaintiff and the accused. For example, in 2008 in the UK, a man was jailed for two years for transmitting Hepatitis B virus to his girlfriend (Mohanty, 2009). However, to date, there is limited literature on the criminal prosecutions of individuals for passing on other sexually-transmitted infections, such as gonorrhoea and chlamydia. A great deal depends upon how the laws of the country are drafted. For

example, in England, an often quoted case is that of *R v Clarence* which related to Clarence transmitting gonorrhoea to his wife during consensual sex. Clarence was charged with inflicting grievous bodily harm and assault occasioning actual bodily harm under S20 or S47 of the Offences against the Person Act 1861. However, he was found not guilty by an 11:4 verdict on the basis that although he knew that he was infected and had not told his wife, she had agreed to have sex with him. It has been suggested that he might have been successfully prosecuted under S23 of the same act that prohibits 'maliciously administering any poison or other destructive or noxious thing' (Orr, 1989).

Apart from HIV, most prosecutions for the transmission of sexually-transmitted infections, especially in USA, tend to occur under tort law (Mekel, 2001). In addition, many of these cases are brought against rich and famous people with a view to obtaining an out of court settlement to prevent the case coming to trial or a large claim from the defendant's insurance company (Eidsmoe & Edwards, 1999).

Malicious transmission of microbial diseases

The deliberate transmission of microbial diseases is sometimes referred to as 'bioterrrorism'. However, the actions of a lone person with a grudge compared with a terrorist organisation or rogue government are usually somewhat different in purpose and scale. It is often stated in the popular press that it would be easy to create and use microbes as biological weapons. However, in practise, without access to dedicated laboratory facilities it would be extremely difficult for people to culture and deliver pathogenic organisms to their target without becoming infected themselves or unintentionally infecting those living nearby and therefore alerting the authorities before the intended 'release date'. Even government organisations with sophisticated containment laboratories sometimes allow their pathogens to escape. For example, in 1979 an outbreak of anthrax in Sverdlovsk in Russia was caused by the accidental release of spores from a military microbiological establishment (Jackson *et al.*, 1998). With the exception of smallpox, which has been eradicated from the world, all the micro-organisms associated with potential bioterrorism also exist as natural infections. One of the most important aspects of microbial forensics is therefore to identify pathogens, distinguish malicious use from a natural outbreak and identify the source of infection and transmission chain (Budowle *et al.*, 2005). This also needs to be done quickly because humans and their domesticated animals and food crops are transported around the world in huge numbers every day, allowing rapid spread of a potential pathogen.

There are few recorded instances in which individuals have intentionally and successfully used microbes to harm others and in most of these cases, the perpetrator had specialist knowledge and access to both facilities and the organisms. For example, when a group of laboratory workers in USA contracted dysentery after eating muffins left as a 'gift' in their canteen there was an instant suspicion that a fellow worker was responsible (Kolavic *et al.*, 1997). This was because the dysentery was caused by *Shigella dysenteriae* type 2 – a relatively rare human pathogen in USA – and apart from the laboratory workers, no one else was infected. Furthermore, pulse-field gel electrophoresis demonstrated that the workers were infected with a strain identical to that stored in the laboratory's own (unsecured) freezer.

The Rajneesh cult in USA was more 'successful' in its use of *Salmonella enterica* var *typhimurium* to cause mass food poisoning and might never have been detected but for the testimony of disaffected cult members several years later. The cult was attempting to influence the outcome of a local election by ensuring that sufficient members of the electorate were too ill to vote. Cult members contaminated the salad bars of local restaurants with bacteria they had obtained legitimately through their own state-

licensed medical laboratory. *Salmonella enterica* var *typhimurium* is not usually associated with large scale food poisoning and there were several aspects of the way in which the outbreak developed that were, in hindsight, suspicious (Morse & Kahn, 2005; Wheelis & Sugishima, 2006). However, this bacterial species had been associated with a food poisoning outbreak elsewhere in the state earlier in the year and the Rajneesh cult never made its involvement known. Somewhat foolishly, cult members retained stocks of the bacterium in their commune laboratory so when a police investigation began it was possible to demonstrate that it was the same as that which caused people to become ill (Török *et al.*, 1997).

The need for specialist knowledge is exemplified by the unsuccessful attempt by the Aum Shrinikyo cult in Japan to disseminate anthrax in 1993. The cult was able to recruit many professionals, including those with scientific and medical training (Olson, 1999). They obtained *B. anthracis* through their contacts and although they had a basic understanding of culture techniques, they did not have enough knowledge to realise that they had sourced a non-pathogenic strain (Keim *et al.*, 2001). Nobody was harmed by the anthrax and the authorities were not even aware of the release until several years later when the cult was investigated for the release of Sarin gas on the Tokyo underground (Wheelis & Sugishima, 2006). The anthrax letters sent to media outlets and two US senators in Washington DC and New York City in 2001 were much more deadly and resulted in 18 people becoming infected and 5 of these died. The subsequent investigation indicated that the letters were the work of someone (or a group) with access to sophisticated equipment and a pathogenic strain of anthrax. At present, the contamination of the letters is generally believed to have been the work of a single person who worked at the US Army Medical Research Institute of Infectious Diseases (USAMRID). He had both the specialist knowledge and the access to the pathogens but as he committed suicide in 2008 he was never tried for the crimes (Enserink & Bhattacharjee, 2008; Koblentz & Tucker, 2010). Molecular typing established that the anthrax belonged to the Ames strain (RMR-1029) that was developed in USA and kept at USAMRID (Bhattacharjee & Enserink, 2008; Enserink, 2008). Bomb pulse dating demonstrated that the spores were formed at some point in the two years before they were posted in the envelopes (Tuniz *et al.*, 2004). Therefore, they were not derived from an old stock that had somehow got onto the 'black market'. Furthermore, isotope analysis indicated that the bacteria had grown in a medium containing water from the north eastern United States (Ember, 2006; Kreutzer-Martin & Jarmen, 2007) and this provided further evidence that the anthrax was not only a US strain but it was cultured in the USA.

Although most published instances on bioterrorism relate to the use of human pathogens and to a lesser extent domesticated animals (Wheelis *et al.*, 2006), plant pathogens could also theoretically be spread maliciously to affect a farmer's livelihood or the economy of a country (Stack *et al.*, 2010). Fletcher *et al.* (2006) have therefore recommended that microbial forensic protocols should be developed that could be used to quickly identify plant pathogens and distinguish instances in which they have been deliberately spread from natural outbreaks and trace their source.

Microbes as indicators of body fluids

There are established tests for the detection of body fluids such as blood, saliva, and semen in forensic samples (Gunn, 2009). However, sometimes these are not sufficiently precise for certain investigations. For example, some of the tests cannot reliably distinguish vaginal fluid and saliva, which could be an important consideration in corroborating allegations of the sequence in which events took place during a sexual assault. When a sample tests positive for both semen and vaginal fluid then this would indicate that

vaginal intercourse had taken place. The vaginal microflora consists of a variety of bacteria but is dominated by a few species belonging to the genus *Lactobacillus* (Jespers *et al.*, 2012). It was once suggested that every woman had a unique bacterial flora (Redono-Lopez *et al.*, 1990) but this is now not thought to be the case (Lamont *et al.*, 2011). However, there is evidence that the vaginal microbiome does differ between ethnic groups (Ravel *et al.*, 2011). Fleming & Harbison (2010) and Akutsu *et al.* (2012) suggest that detecting genetic markers from *Lactobacillus crispatus* would provide a reliable indicator of vaginal fluids but disagree on the suitability of other bacterial species for this purpose. By contrast, Benschop *et al.* (2012) retrieved *Lactobacillus crispatus* DNA from the hands, groin, and penis of volunteers and state that no single species is a reliable indicator and it would be better to use microarray analysis to detect a number of species. Nakanishi *et al.* (2009) have suggested that the detection of oral streptococci would be a reliable means of identifying stains as saliva. In a small study group they used PCR to reliably identify *Streptococcus salivarius* and to a lesser extent *Streptococcus mutans* in saliva but neither species were found in vaginal swabs. However, both species do colonise the vagina, albeit their prevalence tends to be low (Rabe *et al.*, 1988).

Blood can usually be identified unequivocally but there can be doubts about how a stain was caused (James *et al.*, 2005). For example, it can be difficult to distinguish very small stains resulting from impact spatter from those that result from expirated blood. This can be important because a person who comes to the aid of a dying trauma victim might acquire a fine mist of expirated blood on their clothing. However, an assailant could acquire similar stains while carrying out an assault. Expirated bloodstains typically contain bubbles that are absent from impact spatter but these may not be apparent if the blood falls onto an absorbent surface such as cotton clothing (James *et al.*, 2005). Expirated blood is, however, often mixed with saliva and in the process becomes contaminated with salivary bacteria. The streptococci which dominate the oral microbiome do not usually survive for long in the environment (Tagg & Ragland, 1991). However, under experimental conditions the DNA from these bacteria has been detected in seeded blood placed on fabrics for at least 2-3 months (Donaldson *et al.*, 2010; Power *et al.*, 2010). This approach to analysis appears to be potentially useful, but is still at the early stages of development. It is also worth noting that tests for oral streptococci could not be used to identify stains caused by blood that had been sneezed up (Donaldson *et al.*, 2010). This is because the nose contains a quite different microbial composition to that present in the saliva (Lemon *et al.*, 2010) and therefore a different set of DNA primers would be required to identify them.

Soil microbes as forensic indicators

Soil is a useful forensic indicator that can link a person, animal, or plant to a locality (Tibbett & Carter, 2008; Fitzpatrick *et al.*, 2009; Ruffell, 2010). Analyses are usually performed on the mineral and chemical composition of soils but their effectiveness is sometimes limited by the need for relatively large amounts of material and the lack of soil databases (Zala, 2007). All soils contain an extremely diverse microbial community but most species cannot be cultured in the laboratory. With the development of molecular techniques, this is no longer a problem and there are an increasing number of studies investigating the potential of soil microorganisms as potential indicators. Most of these studies have been undertaken using 16-S ribosomal RNA gene sequencing using terminal restriction fragment length polymorphism analysis (T-RFLP) (Smalla *et al.*, 2007; Macdonald *et al.*, 2011; Quaak & Kuiper, 2011) or amplicon length heterogeneity-polymerase chain reaction (ALH-PCR) (Moreno *et al.*, 2006; 2011). The abundance of soil microbes means that very small sample sizes are required; also these techniques are relatively cheap and can be

automated. The results to date have been promising (Hill *et al.*, 2007; Sensabaugh, 2009) but the reliability of this approach could be affected if there are major changes in the soil microbial community over short distances and throughout the seasons. Lenz & Foran (2010) have therefore suggested targeting a more restricted group of soil microbes such as the nitrogen fixing rhizobia.

CONCLUSION

This review has demonstrated how the study of microorganisms can contribute to forensic investigations in a variety of ways from the identification of body fluids to tracing the source of a bioterrorist incident. However, much of the work is still at the early stages of development. For example, although it is possible that the human microbiome differs between ethnic groups, there have been relatively few published studies to date and more information is required on how an individual's microbiome varies with age, place of residence, diet, and health. There is therefore a need for basic research to validate the suitability of microorganisms as forensic indicators of individuals, localities etc. Similarly, although there are standard protocols for the sampling and identification of microorganisms in clinical diagnostic laboratories, this is usually not the case for the analysis of microbial data in other contexts. Disease diagnosis usually involves identifying the presence of a specific species of pathogen or one of its genetic variants whilst microbial forensics often involves profiling a microbial community. Even in the context of a bioterrorist incident in which a single genetic variant of a pathogen is involved, further analytical tests would be required to determine its origin. This is because research laboratories often share clones of microorganisms and therefore it would not be possible to demonstrate which of them may have been the source of the incident from the pathogen's DNA (Lenski & Keim, 2005). In the case of microbial profiling, there are currently two main approaches that both have their advantages and disadvantages. The most commonly used method is to sequence a single marker, such as 16S ribosomal RNA, but it is probable that 'shotgun sequencing' of the whole microbiome will become more widely used in the near future (Haft & Tovchigrechko, 2012). There is therefore a need to develop standard operating procedures (SOPs) for the collection, analysis, and interpretation of microbial forensic evidence if it is to be acceptable in a court of law. Similarly, there will be a need for quality control (QC) and quality assurance (QA) measures, similar to those employed by medical laboratories (Pitt & Cunningham, 2009) to verify the accuracy of the bacteriological results. The admissibility of microbial forensic evidence in a court of law will depend upon the legal framework of the country concerned. For example, in USA the 'Daubert standard' governs the admissibility of scientific evidence in court. In order to meet the Daubert standard, the evidence must be obtained using a procedure that employs a standardised and validated protocol which has a known error rate. In addition, the procedure must have been published after peer review and considered 'generally acceptable' by the appropriate scientific community (Harmon, 2005). Therefore, whilst forensic microbiology offers enormous potential to a wide variety of criminal investigations, there is a need for a great deal of basic research before this can be realised.

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