Eur J Clin Microbiol Infect Dis (2015) 34:1045–1057
DOI 10.1007/s10096-015-2317-x

ARTICLE

How to optimise the yield of forensic and clinical post-mortem microbiology with an adequate sampling: a proposal for standardisation

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Received: 12 November 2014 / Accepted: 7 January 2015 / Published online: 14 February 2015
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Abstract Post-mortem microbiology (PMM) is an important tool in forensic pathology, helping to determine the cause and manner of death, especially in difficult scenarios such as sudden unexpected death (SD). Currently, there is a lack of standardization of PMM sampling throughout Europe. We present recommendations elaborated by a panel of European experts aimed to standardize microbiological sampling in the most frequent forensic and clinical post-mortem situations. A network of forensic microbiologists, pathologists and physicians from Spain, England, Belgium, Italy and Turkey shaped a flexible protocol providing minimal requirements for PMM sampling at four practical scenarios: SD, bioterrorism, tissue and cell transplantation (TCT) and paleomicrobiology. Biosafety recommendations were also included. SD was categorized into four subgroups according to the age of the deceased and circumstances at autopsy: (1) included SD in infancy and childhood (0–16 years); (2) corresponded to SD in the young (17–35 years); (3) comprised SD at any age with clinical symptoms; and (4) included traumatic/iatrogenic SD. For each subgroup, a minimum set of samples and general recommendations for microbiological analyses were established. Sampling recommendations for main bioterrorism scenarios were provided. In the TCT setting, the Belgian sampling protocol was presented as an example. Finally, regarding paleomicrobiology, the sampling selection for different types of human remains was reviewed. This proposal for standardization in the sampling constitutes the first step towards a consensus in PMM procedures. In addition, the protocol flexibility to adapt the sampling to the clinical scenario and specific forensic findings adds a cost-benefit value.

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Introduction

Post-mortem microbiology (PMM) is an important tool in forensic pathology, assisting to determine the cause and manner of death. This is relevant in unexpected deaths, as a microbiological invasion may cause or contribute to rapid death with minimal or no histological inflammation [1, 2]. PMM also aids in the identification of emergent pathogens, novel presentations of known pathogens, drug resistance or bioterrorism agents [3]. Standardized guidelines for microbiological sampling in different post-mortem scenarios are of public health interest [4]: they assist in the identification of

microorganisms causing death, guarantee the allografts' safety for the recipient of cell/tissue transplants and help in tracking the hazard and identifying bioterrorist pathogens.

Historically, PMM has long been a subject of controversy. Several theories intend to explain false positive post-mortem culture results [2, 5]. One of the difficulties in the interpretation of PMM is the growth of microorganisms that are not necessarily pathogenic. These include microorganisms that are part of the commensal flora, related to bacteremia around the time of death, secondary to post-mortem invasion or translocation from oropharyngeal and gastro-intestinal colonized mucosa, and/or contaminants due to inadequate sampling during autopsy [2].

The difficulties around PMM interpretation have relegated its use as a secondary and almost forgotten strategy in forensic autopsy. However, the implementation of specific sterile sampling techniques at autopsy, the use of molecular techniques, the development of laboratory interpretative criteria and the interconnection between microbiologists, pathologists and medical examiners have demonstrated that PMM has a prominent role in forensic medicine [3, 6–8].

Tissue and cells can only be accepted for transplantation after they have demonstrated to be microbiologically safe [9]. PMM is also a useful resource during the investigation of skeletal remains and mummified bodies [10]. Although there are some PMM sampling protocols in use throughout Europe [5, 7 and http://www.seimc.org], there is still lack of standardization.

The aims of our study are: (1) to present recommendations for microbiological sampling at the most frequent post-mortem scenarios faced by forensic and clinical pathologists, cell and tissue bankers, archeologists and anthropologists and (2) to issue general biosafety recommendations when dealing with these specimens.

Methods

A network of forensic microbiologists, forensic pathologists and forensic physicians from Spain, England, Belgium, Italy and Turkey, all working in the judicial system, was initially set up in 2013. During a 15-month period, the network discussed by email, video conferences and face-to-face sessions. One of the meetings was sponsored by a European Twinning Project. This was a collaboration project between the European Union and Turkey aimed at improving the skills of forensic experts (TR/2008/IB/KH/01). This venture included forensic microbiology activities around the need to standardize the PMM sampling. A literature search and the multi-disciplinary experience helped in shaping a flexible protocol. We herein present the consensus on forensic and post-mortem microbiology reached by the expert panel. The clinical scenarios considered were: (A) sudden death (SD), (B) bioterrorism, (C) cell and tissue transplantation, (D) paleomicrobiology.

Results

In all the scenarios described except for cell and tissue transplantation, the staff should wear protective clothing, disposable gloves, head covering and in certain cases an FFP2 mask. Sterile instruments including disposable scalpels and forceps should be used to collect samples. It is essential that a new kit of instruments is used for each sample [11].

A) Sudden death

General recommendations

The following precautions should be taken in each (forensic or clinical) autopsy:

- The body should be placed in a sealed body bag at 4 °C as soon as possible until the autopsy starts.
- The autopsy should preferably be conducted within 24 hours following death, since microbiological samples should be taken as soon as possible after death.
- The skin can be disinfected with a water-based antiseptic such as chlorhexidine 0.05 % with cetrimonium bromide 0.5 % in water. In case isopropyl alcohol is used, toxicologists should be notified, since this compound can be employed as an internal control in toxicological analyses. Organs' surfaces are seared with a red-hot spatula or soldering iron before sampling [1].
- Blood samples, body fluids and nasopharyngeal exudates are best taken at the beginning of the autopsy. Tissue specimens should be obtained with the organs being *in situ*, prior to evisceration [11].
- Tubes with heparin or oxalate (anticoagulant) or fluoride (preservative) should be avoided, as they are toxic to many microorganisms.
- Depending on the amount of the exudate, this can be collected using a syringe, or polyester or other synthetic (flocked) swabs, avoiding cotton or calcium alginate.
- As a general principle, retrieved samples should arrive at the laboratory within 2 hours when stored at room temperature and within 48 hours between 2 and 8 °C when stored in adequate transport media [12].

Based on most frequent case scenarios of SD in which PMM is requested, we envisioned four practical sub-groups according to the age of the deceased person and the circumstances at autopsy. For each sub-group, a minimum number of samples were agreed on. Group 1 includes SD cases in infancy and childhood (0–16 years) without clinical

symptoms (Table 1); group 2 corresponds to SD in the young (17–35 years) without clinical symptoms (Table 2); group 3 corresponds to SD at any age with clinical symptoms (Table 3); and group 4 addresses traumatic or iatrogenic deaths (Table 4). Tables 1, 2, 3 and 4 present the recommended microbiology sampling sites at autopsy, the quantity of material, the type of containers and a general recommendation for microbiological analyses.

Specimens taken during a forensic autopsy are usually considered proof of evidence and a chain of custody must be maintained at all times [13]. This requires documentation of each step in the handling of the evidence, i.e. from the moment of procuring the sample, to its final storage or disposal. The process should be able to demonstrate that the evidence has not been tampered with. This implies the use of tracking forms, both written and in electronic format. Each member of the staff handling the evidence (mortuary, transporters, administrative and laboratory staff) must sign, time and date the type of transaction or task performed. These registers should guaranty the traceability of all the aliquots and DNA-RNA extracts obtained from the original samples. Records should be securely stored. Although currently the only European official recommendation regarding the chain of custody for legal purposes is aimed at seized drugs (Council Recommendation of 30 March 2004 regarding guidelines for taking samples of seized drugs. Official Journal C086, 06/04/2004 P.0010-0011), it is expected that this rule will soon be extended to other forensic issues.

B) Bioterrorism

Intentional misuse of biological agents can lead to bioterrorism, i.e. transfer of this agent to a third party for harmful purposes [14]. Biological agents have been classified into categories (class A–C) according to their risk for public health, their ease of dissemination and social disruption http://www.bt.cdc.gov/agent/agentlist-category.asp#a [14]. Table 5 provides recommendations on sampling of main class A, B and C microorganisms, according to their clinical presentation [3, 14–16].

C) Cell and tissue transplantation

Reducing the risk of transmission of infectious diseases when implanting/transplanting cell/tissue allografts can be achieved following existing guidelines [9]. To maximize microbiological safety, the medical history and physical examination of the potential donor should be investigated. Serological/molecular tests are performed to exclude infection of the donor with *Treponema pallidum*, human immunodeficiency virus, hepatitis B and C virus and, when relevant, human T-cell lymphotropic virus. In addition, the absence of aerobic and anaerobic bacteria, yeast and filamentous fungi is evaluated on the final product of cell/tissue allografts [17, 18]. Table 6 presents an example of a Belgian sampling protocol tailored to each type of cell/tissue graft [19].

Table 1 Sudden death: in infancy and childhood (0–16 years) without clinical symptoms

Site of sample	Quantity	Transport container or medium	Type of analysis
Nasopharyngeal swab	Two flocked swabs	Medium for bacteriology (Amies medium) (N=1)	Bacteriology (only culture)
		Viral transport medium (N=1)	Virology
Blood (peripheral[a] or central)	3–5 ml	Tube with SPS/sodium citrate	Bacteriology (only culture)
	3–5 ml	Bottle for blood culture (aerobic)	Bacteriology (only culture)
	3–5 ml	EDTA	Molecular tests (bacteriology & virology)[b]
Serum	3–5 ml total blood	Centrifugation with serum activator	Serology & antigenic analyses
Lung	>1–2 cm^3 (2 samples)	Sterile tube / container[c]	Bacteriology/ virology and freeze for molecular tests[b]
Spleen	>1–2 cm^3	Sterile tube / container[c]	Bacteriology and freeze for molecular tests[b]
Myocardium	>1–2 cm^3 (2 samples)	Sterile tube / container[c]	Virology Freeze for molecular tests[b]
Bowel content (faeces)	2–3 ml	Cary-Blair medium or sterile container	Bacteriology
		Viral transport medium	Virology
CSF (cerebrospinal fluid)	2–3 ml	Sterile container[c]	Bacteriology Virology: freeze for molecular tests[b]

[a] Preferably peripheral blood. Blood should be taken after opening the body (from the cavities). Preferential order of places to take blood *in situ*: peripheral femoral→subclavian→carotid→jugular→left ventricle

[b] Molecular tests (bacteriology and/or virology, according to symptoms and autopsy findings)

[c] When viral analyses are required, a second container/tube with sterile saline or a RNA stabilization solution is recommended for tissue samples

Table 2 Sudden death in the young (17–35 years old) without clinical symptoms

Site of sample	Quantity	Transport container or medium	Type of analysis
Blood (peripheral)	3–5 ml	EDTA	Molecular tests (bacteriology & virology)
Serum	3–5 ml total blood	Centrifugation with serum activator	Serology & antigenic analyses
Myocardium	>1–2 cm³	Sterile tube / container	Molecular tests (bacteriology & virology) and freeze
Spleen	>1–2 cm³	Sterile tube / container	Molecular tests (bacteriology & virology) and freeze

Molecular tests (bacteriology and/or virology) aim to detect any pathogen causing myocarditis, according to autopsy findings. When there are mild respiratory or gastrointestinal symptoms previous to death, respiratory samples (nasopharyngeal swabs with viral transport medium and lung) or faeces, respectively, should also been taken. A RNA stabilizer solution should be added to tissue samples.

D) Paleomicrobiology

Anthropologists and archaeologists dealing with the study of mummified bodies/partially degraded corpses should take special preventive measures aimed to: minimize the risk of cross-contamination, DNA degradation and ancient DNA contamination with contemporary DNA; and avoid the risk of acquiring infection during handling. It is reasonable to undertake a risk assessment and an exposure control plan based on the specific features of the archaeological site under study. Any agreed measure should be in place at the beginning of specimen collection. Specific criteria to confirm authenticity and to avoid contamination are required [20].

Manipulation of ancient human tissues should take place in facilities specifically dedicated and physically distant from the extraction area. Instrumentation tools should always be cleaned with bleach (10 % sodium hypochlorite) in between samples. The samples should be immediately placed in sterile containers or tubes at 4 °C, protected from light and humidity, and transported to the laboratory as soon as possible.

More specific recommendations for the collection of the different types of suitable specimens include:

Mummified bodies: The tissue type selection for microbiological sampling is based on integrity. Specimens are acquired by aseptic dissection and stored in desiccated chambers. Samples are pulverized under liquid nitrogen. Whenever possible, 400 mg of pulverized tissue is left available for DNA extraction [10]. DNA is more stable in bones than in soft tissues and this seems to be independent of the specific anatomical origin of the samples [21].

Skeletal remains: Selected sections of skeletal elements can be removed using a rotational cutting tool (Dremel tool) on the lowest setting to reduce heat, in order to avoid DNA denaturing [22]. The bone external and internal surfaces should be first cleaned using a radial saw or a Dremel tool to file about 2–4 cm of bone surfaces. If 0.5 % sodium hypochlorite solution is also used to decontaminate, samples should then be rinsed thoroughly afterwards. The radial saw is not appropriate in small bones, the surface can be cleaned with swabs soaked in sterile water. After decontamination, bones are cut into small fragments and UV (ultra violet light) irradiated on their surface. Before extraction, the fragments are pulverized with a freezer mill or a mixer mill [22].

Tooth powder: Intact teeth should be thoroughly cleaned with sterile water and then UV irradiated (see above). If their surfaces are heavily contaminated, they can be cleaned with a scalpel or decontaminated with 0.5 % sodium hypochlorite and washed with sterile water. The teeth are then longitudinally fractured with a cutting wheel, sectioning the cementoenamel junction to expose the pulp chamber. This is opened and the remnants of the dental pulp (which are powdery in ancient teeth), are scraped off and transferred into sterile tubes in order to be pulverized with a freezer mill [23].

Dental calculus: can be extracted directly from dental pieces using a curette and placed in a 1.5-ml tube. The samples are treated with 500 mL of 4–6 % sodium hypochlorite for 1 minute to eliminate surface contaminants, and then washed three times in double-distilled water to eliminate the sodium hypochlorite [24].

Coprolites: Remove the surface first, then UV irradiate the sample. The core of the coprolites needs to be grounded and rehydrated by immersion in a 0.5 % aqueous solution of trisodium phosphate for 72 h. The ancient DNA is extracted by physical-chemical treatment [25].

Biosafety considerations

In any of the above described scenarios, biosafety rules should always be a priority. Microorganisms can be transmitted through inhalation, ingestion or inoculation (intravenous, subcutaneous, direct contact with skin and mucous membranes). Whether or not an infection will develop depends on the

Table 3 Sudden-unexpected death with clinical symptoms at any age. Recommended and complementary specimens according to suspected infections

Specimens	Site of sample	Quantity	Transport container or medium	Type of analysis
Recommended specimens	Blood (peripheral[a] or central)	3–5 ml	Tube with SPS/sodium citrate	Bacteriology (only culture)
		3–5 ml	Bottle for blood culture (aerobic)	Bacteriology (only culture)
		3–5 ml	EDTA	Molecular tests: (bacteriology & virology)[b]
	Serum	3–5 ml total blood	Centrifugation with serum activator	Serology & antigenic analyses
	Spleen	>1–2 cm³	Sterile tube / container[c]	Bacteriology & freeze for molecular tests[b]
Complementary specimens				
Category of symptoms				
Neurological: Category of symptoms				
Bacterial meningitis	CSF, brain tissue Throat swab[d]	CSF: 2–3 ml; brain: >1–2 cm³ 1 swab	Sterile tube / container[c]; Amies medium	Bacteriology & freeze for molecular tests[b] Bacteriology
Encephalitis and myelitis	CSF, brain tissue	CSF: ≥1 ml; brain: >1–2 cm³	Sterile tube/ container[c]	Virology (freeze if required)
Human form of bovine spongiform encephalopathy	CSF Blood	≥1 ml ≥1 ml	Sterile tube EDTA tube (1 ml)	Genetic and 14.3.3 protein studies Genetic and 14.3.3 protein studies
Septicemia, septic shock Disseminated fungemia	Macroscopically abnormal organs: lung, myocardium, liver, kidney Skin petechiae biopsy[e], adrenals[f] CSF (if fungal infection suspected)	>1–2 cm³ of each tissue	Sterile container[c]	In each sample: Bacteriology and freeze for molecular tests Mycology if suspected or immunosuppression
Respiratory symptoms: Pneumonia and other respiratory infections: tonsillitis, epiglottitis, bronchitis, bronchiolitis, croup, flu, pertussis	Always: -Lung (a representative sample from the lobe/s affected by pneumonia)	>1–2 cm³	Sterile container[c]	In each sample: (Myco-)Bacteriology & freeze for molecular tests[b] Mycology if suspected or immunosuppression
	-Nasopharyngeal & bronchial swabs	2 from each site (one with each medium)	Amies medium. If pertussis: Alginate swabs[g] with Amies; Viral transport medium	Bacteriology Virology (freeze if required)
	-Other swabs from affected tissue	2 (one with each medium)	Amies medium Viral transport medium	Bacteriology Virology (freeze if required)
	Throat swabs (if tonsillitis)	2 swabs (one with each medium)	Amies medium Viral transport medium	Bacteriology Virology (freeze if required)
	Pleural fluid (if empyema)	>1–2 ml	Sterile tube	(Myco-)Bacteriology
Food poisoning and gastroenteritis Bloody faeces or hemolytic-uremic syndrome	Bowel content (faeces) Urine	≥1 ml ≥1 ml	Sterile tube Sterile tube	In each sample: Bacteriology & freeze for molecular tests[b] ELISAs for shiga toxin
Fulminant hepatitis	Liver	>1–2 cm³	Sterile container[c]	Virology (freeze if required)
Bacterial peritonitis	Peritoneal fluid (ascites)	≥1 ml	Tube with medium for anaerobes	Bacteriology
Endocarditis		2 swabs/ fragment	Amies medium	Bacteriology & freeze for molecular tests[b]

Table 3 (continued)

Specimens	Site of sample	Quantity	Transport container or medium	Type of analysis
	Cardiac valve vegetation (swabbing/ biopsy)			
Pericarditis	Pericardial fluid	≥1 ml	Sterile tube	Bacteriology & freeze for molecular tests[b]
	Myocardium	>1–2 cm³	Sterile container[c]	Bacteriology & freeze for molecular tests[b]
Soft tissue infections	Swab or tissue sample of affected muscle	2 swabs; >1–2 cm³	Amies Medium. Muscle: Sterile tube / container	Bacteriology (aerobic & anaerobic) & freeze for molecular tests
Abscesses	Purulent exudate content: Syringe aspiration or swabbing the abscess	As much as possible 2 swabs	Sterile tube Amies medium	Bacteriology (aerobic & anaerobic) & freeze for molecular tests
Genitourinary symptoms:	Urine	≥1 ml	Sterile tube	Bacteriology
Pyelonephritis, cystitis	Kidney	>1–2 cm³	Sterile container	Bacteriology & freeze for molecular tests[b]
Genital infection	Cervical & vaginal swabs Other swabs from inflamed tissue	2 (one with each medium)	Amies medium. For *Chlamydia*: Alginate swabs[g] with Amies	Bacteriology & freeze for molecular tests[b]
Chorioamnionitis	Swab of membranes Amniotic fluid	2 swabs ≥1 ml	Amies medium Sterile tube	Bacteriology (aerobic & anaerobic) & freeze for molecular tests[b] Virology (freeze if required)
Congenital/ perinatal infections: Toxoplasmosis or Encephalitis Congenital syphilis Congenital CMV Meningitis or septic shock in neonates (<3 months) Differential diagnosis with non accidental head injury	CSF & Brain tissue If congenital CMV: Urine, saliva & amniotic fluid (prenatal)	≥1 ml >1–2 cm³ ≥1 ml/1 swab ≥1 ml	Sterile tube Sterile container[c] Sterile tube/viral transport medium Sterile tube	Virology: - CMV: ELISA & viral culture on urine, saliva. PCR on urine, saliva & amniotic fluid - PCR / ELISA exanthematic viruses - Enterovirus PCR Bacteriology: -PCR for *Streptococcus agalactiae, Listeria monocytogenes* - Non treponemal and treponemal assays in CSF/serum Parasitology: -Toxoplasmosis: (*Toxoplasma* PCR & ELISA)
Tuberculosis	Tuberculoma from lung or other organs	>1–2 cm³	Sterile container	Specific *Mycobacterium tuberculosis* culture or PCR
Botulism (oculomotor and pharyngeal paralysis, respiratory failure)	Faeces; exudates ; tissues Suspected food transmitted infections	≥1 ml; >1–2 cm³	Sterile container	Toxin detection, *C. botulinum* toxins PCR
Malaria & other systemic parasitosis	Brain, liver, lung, myocardium	>1–2 cm³	Sterile container	Immunochromatography / ELISA / haemagglutination assays in EDTA blood, *Plasmodium spp.* and other specific parasite PCRs

[a] Peripheral blood. Preferential order of places to take blood *in situ*: peripheral femoral→subclavian→carotid→jugular→left ventricle

[b] Molecular tests: bacteriology and/or virology, according to symptoms and autopsy findings

[c] When viral analyses are required, a container/tube with sterile saline or an RNA stabilization solution for molecular analyses is recommended in tissue samples

[d] A throat swab instead of a nasopharyngeal one is recommended if meningococcal septic shock is suspected

[e] If rash

[f] If bilateral adrenal hemorrhage

[g] For these microorganisms alginate swabs are required in case culture is going to be performed

Table 4 Post-traumatic (either admitted to the hospital or not) or iatrogenic death at any age

Site of sample	Quantity	Container or medium	Type of analysis
Blood (peripheral)	3–5 ml	Tube with SPS/sodium citrate	Bacteriology (only culture)
	3–5 ml	Bottle for blood culture (anaerobic and aerobic)	Bacteriology (only culture)
	3–5 ml	EDTA	Microbiology: (molecular & virology) [a, b]
Serum	3–5 ml total blood	Centrifugation with serum activator	Serology & antigenic analyses
Tracheobronchial swab	2 swabs	Amies medium (N=2)	Bacteriology and antigenic analysis
		Viral transport medium (N=1)	Virology
Lung	>1–2 cm³	Sterile tube / container[b]	Bacteriology and freeze for molecular tests[a]
Spleen	>1–2 cm³	Sterile tube / container[b]	Bacteriology and freeze for molecular tests[a]

[a] Molecular tests: bacteriology and/or virology, according to symptoms and autopsy findings

[b] When viral analyses are required, a second container/tube with sterile saline or an RNA stabilization solution for molecular analyses is recommended in tissue samples

Note: According to medical and surgical procedures, it is necessary to add:

-Tissue/fluid from the involved organ (e.g. brain/CSF after neurosurgery, myocardium after cardiac surgery, etc.)

-Catheter tip (2–3 cm of distal end) from central vein

-Pus from deep wounds using a syringe or a sterile swab for bacteriology once the wound surroundings/skin surface are cleaned of debris

exposure route, the bio-burden, the virulence and the immune status of the host. Since all incoming material is potentially infectious, standard safety precautions should be followed both in the autopsy room and in the laboratory [26]. In case of occupational exposure to infectious agents a written protocol should be in place and a training of laboratory personnel is required. Vaccination of personnel should be considered. Disposal of waste should follow regulatory requirements [15]. Table 7 shows the most common microorganisms, hazardous material and specific protection measures that should be taken in the post-mortem environment [27].

Discussion

The success of PMM depends on the adequacy of the post-mortem sampling protocol and strategy [28]. The forensic pathologist needs to contemplate whether PMM should be carried out or not. There are specific indications where PMM is required: to confirm the presence of an unproven infection; when the cause of death is unknown (sudden or unexpected death); to evaluate the efficacy of antimicrobial therapy in eradicating an infection [28]; during the investigation of malpractice related to an infectious disease; and in the archaeological scenario, where it is helpful to identify infectious diseases in human skeletal and ancient remains, especially in mummified bodies [20]. It is crucial to confirm the absence of microorganisms in cells/tissues retrieved in order to confirm that these are safe for transplantation/implantation.

We envisioned four practical sub-groups of case scenarios of SD which could benefit from PMM. The utility of PMM in SUDI (sudden unexpected death in infancy) and in SD in young adults has been demonstrated in recent reports. In one of them, a potential pathogen was found in 57 out of 116 (49 %) infant cases. The use of a complete protocol of multi-site microbiological investigations was important for the identification of cases where these pathogens were the cause of death [7]. Recent developments in PMM and histopathology have increased the identification of an infectious disease as the cause of death in infants, therefore reducing the number of cases that are regarded as "sudden infant death syndrome—SIDS". In patients aged 0–34 years, PMM detected the organism responsible for the death in 17 out of 23 (73.9 %) cases [29]. PMM is relevant for public health in those fulminating fatal infections requiring notification and/or when there is a need to treat the contacts of the deceased [4] or in the recognition of emerging infections.

Bacterial, fungal and viral infections have been transmitted by different types of cell/tissue allografts [30]. Currently, strict control steps occur at various levels of the cell/tissue banking processes with the aim to identify potentially contaminated/infected donors [30], which include donor selection, physical examination, serology, environmental control during tissue procurement, processing, packaging and final microbiological cultures. To the best of our knowledge, no national or international guidelines have ever addressed post-mortem microbiological sampling methods. So far, they only stated that cell and tissue material should be free from bacteria and fungi. Therefore, the Belgian Superior Health Council aimed at establishing such specific recommendations regarding the type of cell/tissue material, the minimum quantity of material, the culture methods and the specific characteristics of detection and reporting of microbiological culture results for cell and tissue allografts.

In addition, the use of hybridisation, real-time PCR, and sequencing informs novel anthropological and

Table 5 Bioterrorism agents: clinical presentation and sampling of main microorganisms[a]

Bioterrorist agent – Class A	Clinical presentation	Differential diagnostics	Postmortem specimens	Early recognition of the bioterrorist microorganism at the laboratory
Bacillus anthracis	Anthrax – meningitis, hemorrhagic mediastinitis, soft tissue infections, sepsis, disseminated intravascular coagulopathy, hemorrhagic colitis	Herpes simplex encephalitis, pulmonary leptospirosis, *Streptococcus pyogenes* (soft tissue infection, meningitis), *N. meningitidis,* yellow fever	Depending on the clinical symptoms : CSF, blood, respiratory samples (swab/biopsy), skin lesion, urine, faeces	Non-hemolytic ground glass appearance and irregular colonies Gram-positive rods, central to subterminal spores
Yersinia pestis	Community acquired pneumonia, sepsis, disseminated intravascular coagulopathy	Hantavirus, influenza, *N. meningitidis, S. pyogenes,* leptospirosis, yellow fever, *S. pneumoniae*	Bubo fluid, CSF, blood, respiratory samples (swab/biopsy), faeces, urine	Pinpoint colonies at 24–48 hrs, hammered copper or shiny appearance after 48–72 hrs, nonhemolytic, plump gram negative rods
Francisella tularensis	Bronchiolitis, bronchopneumonia, hemorrhagic inflammation of the airways, pleuritis and hilar adenopathy, cervical lymphadenitis	Other agents causing community-acquired pneumonia, plague, mumps diffuse alveolar damage	Lung, liver, spleen, pleural fluid, blood	Small colonies on chocolate agar that looked similar to those of a *Haemophilus* species. No growth on sheep blood agar or MacConkey agar
Smallpox viruses	Diffuse rash	Varicella, measles, rickettsia, enteroviruses, *N. meningitidis*, staphylococcal or streptococcal toxic shock	Blood, skin biopsy[b]	
Viral hemorrhagic fever agents: Filovirus, Arenavirus	Fever, diffuse rash, headache, vomiting, diarrhea, abdominal pain, upper respiratory tract infection, sepsis, disseminated intravascular coagulopathy, hepatitis	Varicella, measles, rickettsia, enteroviruses, *N. meningitidis*, staphylococcal or streptococcal toxic shock, dengue, yellow fever, ECHO viruses, leptospirosis, viral hepatitis,	Blood[c]	
Bioterrorist agent – Class B				
Brucella spp.	Acute infection: generalized symptoms such as fever, headache, sweating, anorexia, muscle pain, weight loss Chronic infection: lesions in liver, bone and spleen	Agents causing fever of unknown origin	Blood, bone marrow, lymph nodes, liver, spleen, CSF	Slow-growing very small Gram-negative coccobacillus, poorly growing on blood agar after 24 hrs, oxidase positive and urea hydrolysis
Coxiella burnetii	Zoonotic disease: contact with goats, sheep and cattle Multiple presentations: hepatitis, pneumonia or fever Acute Q fever: fever, headache, myalgia, arthralgia, cough Chronic Q fever: endocarditis	Pneumonic tularemia, viral hepatitis, atypical pneumonia, agents causing fever of unknown origin	Blood, tissue biopsies, body fluids	Pleomorphic Gram-negative coccobacillus, obligatory intracellular, to be cultured in cell lines (eg. monkey kidney cells)
Alphavirus	Encephalitis, fever with convulsions, coma, rash	Other encephalitis causing agents. In a bioterrorist setting they would be detected in the absence of equine illness and out of their natural habitat	Brain biopsy, CSF, blood, serum	Lipid envelope, ss RNA virus. Antibody detection by ELISA, virus detection by RT-PCR or viral culture
Bioterrorist agent – Class C				
Hantaviruses	Fever, malaise, headache, Hantavirus pulmonary syndrome, acute respiratory distress syndrome, hemorrhagic fever with renal syndrome (Asia), nephropathica epidemica (Europe), acute thrombocytopenia	Agents causing pneumonia, in prodromal phase: agents causing fever of unknown origin. Although a zoonotic disease in their natural habitat, in the bioterrorist scenario, no contact with rodents would be found	Blood, serum, urine, CSF, tissues: lung, heart, kidney, liver, brain and spleen	Pleomorphic ssRNA virus with a lipid-bilayer envelope, detection by means of ELISA, viral culture (E6 cell line) RT-PCR in blood and tissues
Nipah virus	Encephalitis, fever, severe headache and respiratory illness.	Other encephalitis causing agents.	Brain biopsy, CSF, lung, kidney, blood, serum	Genus Henipavirus (Paramyxoviridae). Viral culture: syncytial formation of

Table 5 (continued)

Bioterrorist agent – Class A	Clinical presentation	Differential diagnostics	Postmortem specimens	Early recognition of the bioterrorist microorganism at the laboratory
	Naturally causing outbreaks among people with close contact with pigs	It would be found out of endemic areas and without pig's contact		vero cells. In fatal cases, PCR on CSF and tissues, antigen and antibody detection by ELISA
Flavivirus	(Meningo)-encephalitis, fever, headache Some Flavivirus: hemorrhagic shock syndrome, hepatitis.	Other encephalitis causing agents, and other causes of hemorrhagic shock or hepatitis In the bioterrorist setting found out of endemic areas and not seasonally	Brain biopsy, CSF, blood, serum, liver, bone marrow, spleen	Lipid envelope, ss RNA virus. Antibody detection by ELISA, virus detection by RT-PCR or viral culture

[a] Class A microorganisms are those easily disseminated, with high mortality rate with the possibility to cause public panic. Class B agents are those moderately easy to disseminate, with moderate morbidity and low mortality. Class C includes emerging agents that could be easily engineered for mass dissemination in the future due to their availability, simplicity to produce, high mortality rate and major health impact (http://www.bt.cdc.gov/agent/agentlist-category.asp#a)

[b] A confirmed case of Smallpox should not be autopsied

[c] For post-mortem diagnosis of Ebola, oral swabs (saliva and epithelial cells) in viral transport medium are recommended by the WHO (http://www.who.int/crs/resources/publications/ebola/safely-collect-oral-swabs/en/). Additional samples for some viruses are: throat swabs, urine for Lassa; nasopharyngeal swabs, pleural fluid, urine and tissues for Bunyavirus, etc.

Note: Sampling for *Clostridium botulinum*, also a class A microorganism, is included in Table 3. Other class B microorganisms that could be used as bioterrorist agents and that could be detected at autopsy are: *Burkholderia mallei, Burkholderia pseudomallei, Rickettsia prowazekii, Chlamydia psittaci, Salmonella typhi, Vibrio cholerae,* verotoxigenic *Escherichia coli, Salmonella, Shigella,* Cryptosporidium parvum, *Staphylococcus aureus* Enterotoxin B and Epsilon toxin of *Clostridium perfringens*. Table 3 includes the sampling for those enteropathogens that could be used as biological warfare (epigraph: food poisoning and gastroenteritis) as well as the sampling required for class B agents causing septicaemia or respiratory symptoms

Table 6 Sampling protocol for different cell/tissue allografts (based on [19])

Measure	Skin allograft	Fetal membranes	Musculoskeletal tissue allografts	Cardiovascular allografts	Tympano-ossicular allografts	Cornea/sclera	Reproductive cells	Cells other than haematopoeitic stem cells, donor lymphocytes, cord blood	Haematopoeitic stem cells, donor lymphocytes and cord blood
	Source material	Source material	Source material	Source material	Source/final material	Source material	Source/final material	Source/final material	Source/final material
Material to test	Representative tissue remnants	Representative tissue remnants	Representative sample of musculoskeletal grafts taken during procurement: remnant and/or swab taken from total surface graft	Representative microbiological sample	No sampling in case of 14-day formaldehyde procedure Optional: sample of last storage medium	Tissue remnant or fluid	Gonadal procurement: at procurement or after preparation -Ovaria: rinsing or preparation medium or gonadal tissue remnants -Testes: rinsing or preparation medium -Sperm (allogeneic donation): sperm sample of each donor during selection (before donation) and/or periodically during donations or on indication. Not for partner donation	Transport medium or cell material	Cell suspension (in case no fractionation and cryopreservation) residual amount of erythrocytes or plasma fraction
Quantity	1–2 % of procured skin surface	1–2 % of total membrane surface	1 sample per separately packed allograft	−1 % of total volume of transport medium −20 mL of the last preparation medium		Residual material or 1 % rinsing fluid (≥100 µL)	Gonadal procurement: at each donation, ≥1 % of the total volume of rinsing/preparation medium (add up to 100 µL) or tissue remnants Sperm: ≥1 % of the total amount of untreated sperm, add up to 100 µL	≥1 % of total volume (≥100 µL)	≥1 % of total cell suspension (≥100 µL) Eryhtroyctes or plasma fraction: 1–5 mL
Detection	Distinction low and high virulent species[a] and bioburden, necessary for further actions	Absence of high-virulent microorganisms[a] or extra measures	Absence of high-virulent microorganisms[a] or extra measures	Absence of high-virulent microorganisms[a] or extra measures		Ocular tissues in general are contaminated at retrieval extra measures are possible	Partner donation or autografts: microbiological control to prevent cross contamination and as surveillance culture allogeneic sperm donation: material contaminated with high virulent pathogens[a] is eliminated		
Final material									
Material to test	Representative tissue remnants taken just before final packing	Non-irradiated membranes: representative sample for the t otal of separately packed allografts taken at final packing Irradiated membranes: packed sample of final product	Non-irradiated allografts: tissue remnant and/or rinsing fluid and/or swab taken from total surface Irradiated allografts: packed samples of final product	Representative microbiological sample	No sampling in case of 14-day formaldehyde procedure Optional: sample of last storage medium	Cornea: -Storage medium (cold and warm storage method) and transport medium in case of warm storage method sclera: -Ethanol 70 % OR -last rinsing fluid OR -Cornea storage medium (cold storage method) OR		Adherent cells: supernatans of cell storage medium Pooled supernatans in case of several culture bottles Cells in suspension: cell storage medium after short sedimentation Pooled supernatants in case of several culture bottles	Residual amount of erythrocytes or plasma fraction OR product to be frozen (after adding cryoprotecting solution to final product)

Table 6 (continued)

Measure	Skin allograft	Fetal membranes	Musculoskeletal tissue allografts	Cardiovascular allografts	Tympano-ossicular allografts	Cornea/sclera	Reproductive cells	Cells other than haematopoeitic stem cells, donor lymphocytes, cord blood	Haematopoeitic stem cells, donor lymphocytes and cord blood
	Source material	Source material	Source material	Source material	Source/final material	Source material	Source/final material	Source/final material	Source/final material
Quantity	1–2 % of total skin surface	Non-irradiated membranes: 1–2 % of the total fetal membrane surface Irradiated membranes: -2 packed samples of a batch irradiated fetal membranes in case ≤50 samples/batch -5 % of packed samples of a batch irradiated fetal membranes in case >50 samples/batch	Non-irradiated allografts: 1 sample (tissue remnant and/or swab and/or rinsing fluid for every separately packed allograft Irradiated allografts: -2 packed samples of a batch of ≤50 samples/batch - 5 % packed samples of a batch of >50 samples/batch	−1 fragment per individually packed allograft AND, for cardiac graft - 1 sample of cryopreservation solution taken immediately before definitive final internal sealing of package	≥1 % of total volume (optional)	-Cornea transport medium (warm storage method) OR sclera remnant Cornea: ≥1 % volume of cornea storage and/or transport medium (≥100 µL) Sclera: -Ethanol 70 %: ≥1 % of the volume (final dilution of ethanol 1/10) (≥100 µL) -≥1 % volume of sclera rinsing fluid (≥100 µL)		≥1 % of total volume (≥100 µL)	Final product with cryoprotecting agent: ≥1 % of total volume (≥100 µL)
Detection	Absence of micro-organisms[b]	Absence of micro-organisms[b]	Absence of micro-organisms[b]	Absence of micro-organisms[b]	Absence of micro-organisms[b]	Absence of micro-organisms[b]		Absence of micro-organisms[b]	Absence of micro-organisms[b]

[a] High virulent species: *S. aureus*, *Enterococcus* spp., betahemolytic streptococci, all gram-negatives, sporulating microorganisms (*Bacillus* spp., *Clostridium* spp.), yeast and molds

[b] Exceptional release of allografts is always possible in case of life-saving procedures. This is the responsibility of the tissue bank director

Table 7 Common microorganisms with their hazards in postmortem environment [16, 27]

Microorganism	Hazardous material/action	Contaminated material	Microorganism-specific personal protection
Mycobacterium tuberculosis	Aerosol : splashes, oscillating saw, aspirator	Respiratory samples, gastric lavage, urine, CSF, lesions	High efficiency particulate-air filter (FFP2)
Neisseria meningitidis	Ingestion, aerosol, parenteral inoculation, droplet	CSF, pharynx, blood, saliva	Vaccination (some serogroups) and post-exposure prophylaxis
Hantaviruses	Aerosol : splashes, oscillating saw, aspirator	Blood, respiratory material	High efficiency particulate-air filter (FFP2)
Hepatitis B, C and D	Parenteral inoculation Mucosal contact	Blood and body fluids	HBV Vaccination
Hepatitis A and E	Faeco-oral	Blood, faeces	HAV Vaccination
HIV	Parenteral inoculation Mucosal contact	Blood and body fluids	Post-exposure prophylaxis

paleopathological research. These techniques are an aid in the identification of, for instance, leprosy, malaria, Chagas disease, plague, syphilis, diphtheria or typhoid fever [20]. Moreover, microbiology laboratories performing forensic/post-mortem analyses should perform their own appropriate validation studies on post-mortem samples to evaluate the application of both "in-house" and commercial serological or molecular assays, due to the fact that many commercial assays don't include these specimens in their developmental validation.

PMM contributes to the search for the cause of death in forensic pathology [3], and its strength is enforced when using standardized sampling protocols at autopsy. These sampling protocols should be adapted to the specific post-mortem circumstances. The closer to the moment of death the samples are obtained, the higher the quality and the success rate. To obtain useful microbiological results, sampling should be adequate and thorough. The tests performed need to be selected from a broad portfolio, according to the infection suspected and using cost-benefit criteria. The preferential sites for peripheral blood sampling in PMM differ from those used in routine clinical practice. As an example, blood sampling in routine clinical practice is preferably not performed via the femoral vein because of the high yield of contaminants. At post-mortem, femoral or external iliac blood vessels are sampled *in situ* (during the abdominal time) and after clamping the common iliac vein. Once the microbiological analyses are concluded, the forensic microbiologist and forensic pathologist need to assess the microbiological results obtained in relation to the circumstances of death and to clinical, macroscopic and histopathological findings [5, 7]. In the near future, new technologies such as the analysis of non-nucleotidic biomolecules [31] are promising for their contribution to the success of forensic microbiology.

In conclusion, the sampling protocols proposed here aim to standardize the microbiological sampling for the most frequent situations seen in the daily forensic and post-mortem practice. Wider implementation of post-mortem sampling recommendations will allow a better interpretation of the microbiological results related to forensic/post-mortem cases and a better understanding of the mechanisms and manner of death related to unsuspected infectious diseases in forensic practice.

Acknowledgements *In memoriam* of Mª José Burguete†, a skilled microbiology technician at the Instituto Nacional de Toxicología y Ciencias Forenses. The authors also want to thank Dr. Pedro Manuel Garamendi for all his support for forensic microbiology all along the European Twinning Project TR/2008/IB/KH/01.

Conflict of interest The authors declare no conflicts of interest.

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