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FORENSIC MICROBIOLOGY: A CASE SERIES ANALYSIS

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ABSTRACT

The importance of microbial colonization of human organs in a living body has long been assessed. What still remains unclear are the microbial changes occurring after death, thus leading to the advent of a relatively novel field of research called *"Postmortem microbiology"*. It is applied to several forensic fields such as post-mortem interval assessment and cause of death determination. In this contest, a major limit is thus represented by the correct interpretation of the microbial data and by the actual lack of standard procedures.

Here we propose a standard operative protocol in order to avoid false positives given by contamination (the main problem), *post-mortem* translocation and agonal spread. The protocol was implemented in 36 judiciary autopsies and allowed in the assessment of an infection in 10 cases. The study showed the usefulness of the protocol which also supports the contribution of forensic microbiology, together with clinical, histopathological, biochemical and autopsy data, to the determination of infectious cause of death.

1. Introduction

According to the Human Microbiome Project (HMP), a healthy human body contains ten times more microbes than human cells. In a healthy human body, microbes usually organize in site-specific communities, playing fundamental roles both in human health and disease [1-3]. As death occurs, the failure of the immune system and of the physical barriers allows the proliferation and spread of the microbes throughout the body, starting from the ileocecal area [4]. In order to better understand the microbial changes occurring after death, and thanks to the advent of novel biomolecular approaches (PCR, sequencing, etc.), we can now start to talk about *Human Postmortem Microbiome*, comprehensive of the microbial populations colonizing internal organs and fluids (referred to as *thanatomicrobiome*), and microbes residing on/invading decomposing remains (referred to as *epinecrotic communities*) [5].

The usefulness evidence of cultural investigations in several forensic fields has given rise to a relatively novel research field, called *Postmortem Microbiology*, whose wide spectrum of applications includes: PMI determination, cause of death determination, isolation of microbes as markers of a specific type of death, biocrime, trace evidence, Healthcare Associated Transmission (HAT) diagnosis, sexual abuse evidence, people identification, and population studies [6-12]. However, a consistent problem is related to the correct interpretation of the microbial result. When microbes are isolated from organs and fluids, which are normally sterile during one's life, the cultural result can be interpreted as contamination, agonal spread, *postmortem* translocation or genuine positive: the first three events usually give rise to a mixed growth, while the last one usually produces a pure growth [13,14].

Unfortunately, contamination cannot be avoided at all, but can be decreased to levels similar to those found on samples obtained in life (4-6%) if stringent precautions are taken [15].

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Agonal spread of microbes should occur during the process of dying or during resuscitation procedures as a consequence of the ischaemic damage induced on mucosal surfaces, but is in fact more a theoretical than practical concept since, as shown in several studies, it doesn't seem to occur. *Postmortem* translocation, referred to as the bacterial migration from the mucosal surfaces into the blood and tissues starting from the ileocecal area, can be avoided if corpses are stored at 4°C prior to autopsy. Regarding the contamination problem, although the scientific community agrees on the importance of carrying out the cultural investigations according to asepsis procedures, there's still a lack of consensus about it. It is thus desirable to propose a single, universally accepted, operative protocol in order to standardize the samples collection and cultural procedures, and to make the interpretative data uniform wherever the cultural investigations are carried out.

The aim of the present study is to report the microbiological analyses carried out on samples obtained from a total of 36 cases with several causes of death in order to highlight the role of forensic microbiology as a tool to determine cause and manner of death, and to clarify implementing rules and timing by providing a practical example of operative protocol.

2. Methods

The study was carried out on samples of cardiac and femoral blood, pericardial and pleural fluids, lung, liver and spleen tissues, obtained by means of standard collection techniques from 36 corpses during autopsy. The sampling was carried out according to the rules of asepsis indicated by Riedel *et al.*, and to the procedures issued by the European Congress of Clinical Microbiology & Infectious Diseases (ESCMID) Study Group for Forensic and *Postmortem* Microbiology (ESGFOR), in order to exclude contamination or *postmortem* translocation of microbes: body storage at 4°C; samples collection within 24h and 48h from death, prior to evisceration of the body; use of appropriate collection media; sterilization of the surfaces of the body sites chosen; use of sterile tools; immediate transfer of the samples collected to the microbiology laboratory [16].

Samples collection and microbiological analysis

First, the corpses' skin was sterilized with Betadine 10% on the sites of incision and peripheral blood draw (Figure 1). Before incision, femoral blood was obtained using a disposable syringe, and then collected in proper blood culture media. Once opened, the corpses' pleural and pericardial fluids and cardiac blood were obtained using disposable syringes after the sterilization of, respectively, serous membranes and cardiac surface with a Bunsen heated spatula, and then collected in proper blood culture media. Lung, spleen and liver samples were obtained after the sterilization of their surface with a Bunsen heated spatula, and then collected in disposable tubes (Figure 2). All samples were stored at 2-8 °C and sent to the microbiology laboratory within a maximum of 48h. Blood and pericardial and pleural fluids were cultured for up to 14 days on aerobic and anaerobic media, in order to isolate bacteria and fungi, using the Bactec FX blood culture system (Becton, Dickinson, Cockeysville, MD), while tissue samples were homogenized (24,000 × g/min) in sterile PBS. Fluid samples in which the Bactec fluorescence system detected growth, and homogenized tissue samples were then cultured on selective and nonselective media (Columbia blood agar, Cetrimide agar, Mannitol salt agar, MacConkey agar, Chocolate agar, HE agar, SS agar and Sabouraud agar).

Incubation was carried out under aerobic atmosphere–with the exception of Chocolate agar- incubated under 5% CO_2–at 37 °C for up to 7 days.

The microorganisms were identified with BD Phoenix™ systems. All investigations were performed with PMIC/ID88 panel for Gram positive organisms, SMIC/ID11 panel for Streptococci and NID and MIC502 for Gram negative organisms.

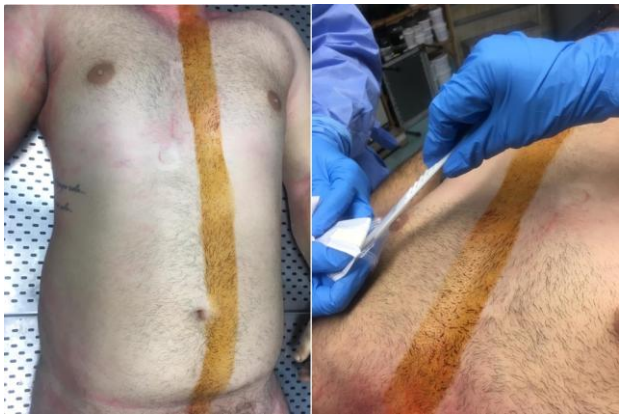

Figure 1. Sterilization of corpses' skin on the sites of incision.

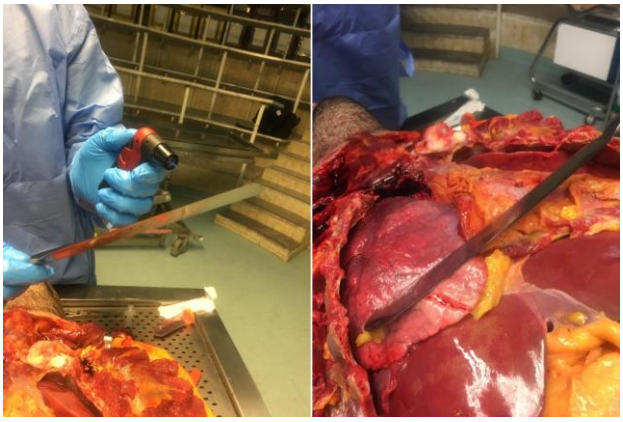

Figure 2. Sterilization of serous membranes and cardiac surface with a Bunsen heated spatula.

3. Results

The microbiological analyses have been carried out on samples obtained from 36 corpses. As shown in Table 1, on samples collected from 24 cases, no microbial growth was detected. This result represents our negative control, since these samples were obtained from people who died traumatic, non-infective deaths.

Samples obtained from 2 of the remaining 12 cases were considered contaminated: case 1 is a dismembered body found many days after a flood; case 14 is a charred body with destruction of the head and thoracic and abdominal walls. These cases showed a mixed growth of environmental bacteria as *Bacillus* spp, Enterococcus spp and *Staphylococcus* spp.

Cultural results on samples obtained from cases 16, 20, 24 and 25, confirmed the microbiological data reported in the clinical records.

The analysis showed infections by multidrug resistant bacteria associated with healthcare (*Acinetobacter baumannii*, *Pseudomonas aeruginosa* and *Staphylococcus aureus*).

The cultural results on samples obtained from the remaining 6 cases (n. 4, 12, 27, 32, 35 and 36), also supported by other *postmortem* findings (i.e. histopathological data), instead allowed us to determine the cause of death *ex novo*.

Case n.	Case n.	Case n.	Case n.	Case n.	Case n.	Case n.
1	M	65	Drowning	Putrified - dismembered	Pos	*Streptococcus* spp, *Bacillus* spp, *Pseudomonas* spp	Cardiopulmonary failure due to drowning
2	M	31	H2S intoxication	"Fresh"	Neg		Acute pulmonary oedema and cardiac arrest due to intoxication
3	M	39	H2S intoxication	"Fresh"	Neg		Acute pulmonary oedema and cardiac arrest due to intoxication
4	F	54	Health care	"Fresh"	Pos	*Acinetobacter baumannii*	Septic shock
5	M	0	Stillbirth	"Fresh"	Neg		Intrauterine hypoxia
6	M	35	Suicide (firearms)	"Fresh"	Neg		Skull and brain trauma
7	M	54	Road accident	"Fresh"	Neg		Skull fratures with massive subdural haemorrhage and brain contusion
8	M	43	Road accident	"Fresh"	Neg		Thorax and abdominal trauma with lung contusion and liver lesions
9	M	64	Hypothermia	"Fresh"	Neg		Cardiopulmonary failure due to severe hypothermia
10	M	55	Homicide (firearms)	"Fresh"	Neg		Skull and brain trauma
11	M	36	Road accident	"Fresh"	Neg		Skull and brain trauma with spleen lesions
12	F	66	Road accident - hospitalization	"Fresh"	Pos	*Proteus mirabilis*	Septic shock
13	M	61	Suicide (firearms)	"Fresh"	Neg		Skull and brain trauma
14	M	78	Suicide (burning)	Carbonized	Pos	*Bacillus* spp, *E. coli*, *Staphylococcus* spp, *Enterococcus* spp.	Severe hypovolemic shock
15	M	32	Sudden cardiac death	"Fresh"	Neg		Myocardial infarction
16	F	64	Health Care	"Fresh"	Pos	*Acinetobacter baumannii*	Septic shock
17	M	0	Stillbirth	"Fresh"	Neg		Intrauterine hypoxia
18	M	63	Road accident	"Fresh"	Neg		Skull fratures with massive subdural haemorrhage and brain contusion
19	M	55	Sudden cardiac death	"Fresh"	Neg		Miocardial ischaemia
20	F	72	Strangulation - hospitalization	"Fresh"	Pos	*Pseudomonas aeruginosa*	Multiorgan failure due to hypoxic damage and sepsis
21	M	56	Health care	"Fresh"	Neg		Hemorrhagic shock
22	M	79	Road accident	"Fresh"	Neg		Skull fratures with massive subdural haemorrhage and brain contusion
23	M	46	Homicide (burning alive)	"Fresh"	Neg		Severe hypovolemic shock
24	M	57	Health care	"Fresh"	Pos	*Staphylococcus aureus*	Septic shock
25	M	69	Health care	"Fresh"	Pos	*Pseudomonas aeruginosa*	Septic shock
26	M	39	Health care	"Fresh"	Neg		Haemorrhagic shock
27	M	49	Drug addict	"Fresh"	Pos	*Streptococcus pyogenes*	Septic shock
28	M	29	Homicide (firearms)	"Fresh"	Neg		Skull and brain trauma
29	M	75	Accident (burning)	"Fresh"	Neg		Severe hypovolemic shock
30	M	33	Road accident	"Fresh"	Neg		Haemorragic shock due to aortic rupture
31	M	35	Hanging	"Fresh"	Neg		Cardio-pulmonary failure due to acute asphyxia
32	F	29	Cesarean section	"Fresh"	Pos	*Escherichia coli*	Post-partum haemorrhage and sepsis
33	M	61	Work accident	"Fresh"	Neg		Hemorrhagic shock
34	M	82	Hanging	"Fresh"	Neg		Cardio-pulmonary failure due to acute asphyxia
35	F	73	Gastroenteritis and sepsis	"Fresh"	Pos	*Salmonella Enteritidis*	Septic shock
36	F	59	Sudden death in alcoholic	"Fresh"	Pos	*Pseudomonas aeruginosa*	Septic shock

Table 1. Summary of the microbiological results and causes of death of each case, in relation to circumstances of death and status of the body at autopsy. Red: case with contaminations; Green: case in which post-mortem microbiology confirmed ante-mortem clinical data; Blue: case with positive post-mortem microbiological analysis and without ante-mortem microbiological data.

In cases 4 and 12 the post-mortem microbiological analysis resulted positive for *Acinetobacter baumannii* and *Proteus mirabilis*, respectively, and both bacteria were multidrug resistant suggesting, together with the data of clinical records (i.e. temperature, leukocytosis, increase of protein C reactive and procalcitonin), that the infections were related to healthcare. Instead, the microbiological analysis of cases 27, 32, 35 and 36 resulted positive for bacteria not healthcare-related showing sensibility to drugs (*Streptococcus pyogenes*, *Pseudomonas aeruginosa*, *Escherichia coli* and *Salmonella Enteritidis*) and identified the cause as septic shock.

4. Discussion

Forensic microbiology is a relatively novel field of research relying on cultural investigations carried out on internal organs and fluids collected *post-mortem*, in order to obtain information which can be useful in a medico-legal contest. The major concern is represented by the interpretation of the cultural result: contamination represents a problem since it cannot be entirely avoided, although it can be reduced to levels very close to those found on samples obtained *antemortem* if stringent precautions are taken; *post mortem* translocation can be avoided if corpses are stored at 4°C prior to autopsy; agonal spread is more a theoretical concept since it doesn't actually occur [4].

Contamination, which represents the main problem in *post mortem* microbiological analyses, leads to unreliable results and, therefore, to difficulties related to ascertaining the cause of death. In order to avoid this, the pathologists can implement suitable activities aimed to obtain asepsis.

Such prevention activities relate to the time elapsed before the autopsy is executed (body storage at 4°C) and between death and samples collection (which must be obtained within a maximum of 48 h from death, prior to evisceration of the body), the sampling collection procedures (sterilization of the surfaces of the body sites chosen, use of appropriate collection media and sterile tools), the post-sampling collection management (storage at 2-8°C) and the time within which the microbiological analysis is performed [17]. It should also be highlighted that these actions are useful in order to prevent *post mortem* translocation.

Once contamination is excluded, agonal spread and *post mortem* translocation, which are sources of false positives, a pure growth of a pathogen can be considered a genuine positive, usually associated with infection/sepsis. However, while the presence of a pathogen in the blood is usually linked to a significant disease in life, this concept isn't always true when a pathogen is isolated after death. Bacteraemia can in fact occur in life even in patients with an unascertained cause of death, meaning that the pathogens isolated after death could just be contributing, not aetiological, factors of death (especially if cultural results are not corroborated by autopsy and histological findings). This means that, in order to link a pathogen isolation to an infectious/septic cause of death, it is important to correlate, when possible, *ante mortem* and *post mortem* cultural results together with the clinical history of the patient, and biochemical, autopsy and histological findings [18-21]: a multidisciplinary approach is thus required. Nevertheless, it must be remembered that in some cases the autopsy and routine histology can be unspecific and unconvincing [18], meaning that other investigations such as *post mortem* microbiology and biochemistry too prove fundamental to better define the *post mortem* diagnosis of sepsis.

The present study was performed using asepsis procedures suggested by the European Congress of Clinical Microbiology & Infectious Diseases (ESCMID) Study Group for Forensic and *Postmortem* Microbiology (ESGFOR). Each case was then analyzed taking into account all the findings obtained from circumstantial data, clinical records, autopsy, histological and microbiological investigations, together leading to establish the cause of death.

Cultural investigations produced negative *postmortem* microbiological results in 24 cases, according to *antemortem* data and autopsy and histological findings as well. The absence of microbial growth confirms the adequacy of the protocol used in order to avoid contamination, and supports the importance of performing a correct *post mortem* body management and sampling collection.

Microbiological results from only 2 cases were considered as contaminated, also according to the conditions of the bodies at the time of their discovery.

In both cases, the alteration of the body's integrity, due to the circumstances of death and to the conditions of the bodies at the time of discovery, encouraged contamination by environmental microbes, as demonstrated by the occurrence of a mixed growth.

Genuine positive results were observed in 10 cases in which the autopsy and/or histological analyses demonstrated infectious signs as well. Specifically, the *post mortem* microbiological analyses confirmed the *antemortem* ones, according to clinical records, in 4 cases; septic shock was considered as cause of death in 3 cases, while in case n. 20 sepsis was considered a contributing factor to the occurrence of death, together with hypoxic damage due to asphyxia.

Even more interesting were the results obtained from the last 6 cases, all lacking *antemortem* data, suggesting an infectious disease. In these cases, the *post mortem* microbiological analyses gave rise to pure microbial growth, proving critical for defining septic shock as the cause of death in 5 cases. In case n. 32, infection was considered as a contributing factor to the occurrence of death, together with the systemic effects of *postpartum* hemorrhage.

These findings confirm that the association of *post mortem* cultural results with *ante mortem* clinical data (if available) and other *post mortem* findings, are often useful to confirm or diagnose an infective cause of death. In this setting, many researchers have always stressed the importance of implementing the *post mortem* sampling collection during the autopsy for microbiological analyses as an additional laboratory investigation which could prove very useful in the forensic assessment of sudden cardiac death, sudden unexplained death and sudden infant death [22-25].

The present study thus led to important considerations:

1) It is fundamental to perform a correct sampling relying on rules of asepsis in order to avoid contamination;

2) Microbiological analyses are useful in the forensic assessment of the cause of death if corroborated by a supportive clinical history of the patient (if available) and/or by necropsy, histological and biochemical findings.

Nevertheless, it must be highlighted that a large part of the forensic microbiology issue is relative to the lack of standard procedures for the collection and analysis of samples, thus, a general effort is needed to obtain standardized protocols in order to make the interpretative data uniform and to allow this recent field of research to show all its potential.

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