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Genetic Diversity of *Mycobacterium avium* Isolates Recovered from Clinical Samples and from the Environment: Molecular Characterization for Diagnostic Purposes▽

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Isolation of *Mycobacterium avium* complex (MAC) organisms from clinical samples may occur in patients without clinical disease, making the interpretation of results difficult. The clinical relevance of MAC isolates from different types of clinical samples ($n = 47$) from 39 patients in different sections of a hospital was assessed by comparison with environmental isolates ($n = 17$) from the hospital. Various methods for identification and typing (commercial probes, phenotypic characteristics, PCR for detection of IS*1245* and IS*901*, sequencing of the *hsp65* gene, and pulsed-field gel electrophoresis) were evaluated. The same strain was found in all the environmental isolates, 21 out of 23 (91.3%) of the isolates cultured from urine samples, and 5 out of 19 (26.3%) isolates from respiratory specimens. This strain did not cause disease in the patients. Testing best characterized the strain as *M. avium* subsp. *hominissuis*, with the unusual feature that 81.4% of these isolates lacked the IS*1245* element. Contamination of certain clinical samples with an environmental strain was the most likely event; therefore, characterization of the environmental mycobacteria present in health care facilities should be performed to discard false-positive isolations in nonsterile samples, mainly urine samples. Molecular techniques applied in this study demonstrated their usefulness for this purpose.

The clinical relevance of *Mycobacterium avium* complex (MAC) has increased in recent decades because of the rising population of immunocompromised individuals due to longer life expectancy, immunosuppressive chemotherapy, and AIDS pandemia (28). In AIDS patients, MAC is the most common nontuberculous mycobacteria causing disease, especially in developed countries, and usually produces disseminated infections (8, 16). However, MAC also causes infection in immunocompetent individuals with underlying pulmonary disease and in children with lymphadenitis or cystic fibrosis (10, 14, 16).

Mycobacterial species comprised in MAC have undergone reorganization based on biological, serological, and molecular characteristics (Table 1). The most important species causing disease in humans can be differentiated using PCR-based methods for the detection of the presence/absence of insertion sequences (IS): *M. avium* subsp. *avium* (37) presents IS*901* (7, 21, 27) and low copy numbers of IS*1245* (13, 17), *M. avium* subsp. *hominissuis* (25) usually harbors high numbers of IS*1245* but no IS*901* (25), and *Mycobacterium intracellulare* (32, 42) presents neither IS*901* nor IS*1245* (2). Other MAC species include *M. avium* subsp. *paratuberculosis*, whose involvement in Crohn's disease is still under discussion (9), and *M. avium* subsp. *silvaticum* (37).

In spite of the environmental colonization and rising prevalence of disease due to members of MAC, the epidemiology of infection is still not clear. The routes of transmission have not been unambiguously identified, and human-to-human transmission has not been demonstrated. Mycobacteria belonging to MAC have also been detected in a wide range of animal species, including birds (mainly *M. avium* subsp. *avium*) (27), pigs (more frequently *M. avium* subsp. *hominissuis*), (19), and small vertebrates (11).

The environment is the most likely source of infection for humans, as *M. avium* is considered a ubiquitous organism and has been isolated from water (1), soil, and dust (15). Water distribution systems have been reported as a possible source of infection in hospitals, homes, and commercial buildings (1, 41); moreover, hospital water can also contaminate samples of uninfected patients, making the clinical significance of the MAC isolation unclear (38).

Current diagnosis is based on probes or PCR tests that occasionally are not able to discriminate between MAC species or subspecies. Several molecular biology techniques can be used for epidemiological studies in order to establish measures to control the dissemination of the infection. For this purpose, restriction fragment length polymorphism (RFLP) using IS*1245* as a probe (40) has been widely used; however, the effectiveness of IS*1245* RFLP is limited in *M. avium* strains harboring a low number of IS*1245* copies.

Pulsed-field gel electrophoresis (PFGE) has also been shown to be a useful technique for assessing the relatedness of strains among MAC (24). Nevertheless, PFGE requires a large amount of bacterial DNA for good visualization of the patterns. Other characterization techniques include sequencing of

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TABLE 1. Characteristics of the most common MAC species causing human disease

Strain	Serotypes	Virulence for birds	IS*901*	IS*1245*	*hsp65* code(s)[a]	Reference(s)
M. avium subsp. *avium*	1–3	Virulent	+	+	4	25, 37
M. avium subsp. *hominissuis*	4–6, 8–11, 21	Partially virulent	−	+	1–3, 7–9	25
M. intracellulare	7, 12–20, 22–28	Avirulent	−	−	10–12, 14	32

[a] According to Turenne et al. (39).

the 65-kDa heat shock protein gene (*hsp65*) (29, 39) and of the internal transcribed spacers (ITS) (26).

The aim of this study was to characterize the environmental strains of MAC present in a hospital using different molecular tests (IS*1245* and IS*901* detection, *hsp65* sequencing, and PFGE analysis) and to compare them with clinical isolates from patients from the same hospital in order to evaluate the possibility of nosocomial infections or contamination of samples.

MATERIALS AND METHODS

A total of 64 isolates belonging to MAC from a public hospital were analyzed. This hospital covers a population of 525,000 inhabitants and is located in Madrid (Spain). Forty-seven isolates were recovered from clinical samples obtained from patients from different sections of the hospital. The other 17 isolates were cultured from several environmental sources (tap water from various floors of the hospital and containers for the collection of urine samples). All the isolates were cultured in the period between 2002 and 2005.

Clinical *M. avium* isolates. Forty-seven MAC isolates were recovered from 12 AIDS patients and 27 patients not infected with human immunodeficiency virus (HIV). Initially, all patients showed clinical syndromes compatible with mycobacterial diseases (pneumonia, meningitis, pericarditis, etc.) though active mycobacterial infection was afterwards confirmed in only 13 patients based on clinical criteria. The isolates were processed according to Tacquet and Tison (34) and cultured in Coletsos medium (BioMedics S.L., Tres Cantos, Madrid, Spain). Among these 47 isolates, 13 were sequential isolates cultured from identical or nonidentical sites from five patients (patient numbers 17, 18, 19, 35, and 36), and the rest were single isolates from 34 patients. Thirty-eight isolates were cultured from admitted patients, while the remaining nine isolates were obtained from nonadmitted patients. Most of the isolates were cultured from nonsterile sites (urine, $n = 23$; sputum, $n = 19$) except for five isolates (blood, $n = 3$; liver biopsy, $n = 1$; bone marrow, $n = 1$).

Environmental *M. avium* isolates. Eleven isolates were cultured from the intermediate containers used for the collection of urine samples in disabled patients and six were from tap water taken from the different patient's rooms at the hospital. Fifty milliliters of water was collected from the water taps of the patients' rooms into sterile tubes; the intermediate containers were cleaned following the standard procedure and then rinsed with 50 ml of sterile water that was afterwards collected into sterile tubes. These samples were centrifuged at 3,500 rpm for 30 min; the pellet was decontaminated according to Tacquet and Tison (34) and centrifuged again at 3,500 rpm. The pellet was then inoculated into Löwestein-Jensen medium supplemented with amphotericin (400 mg/liter), nalidixic acid (35 mg/liter), and lincomycin (2 mg/liter) and into Coletsos (BioMedics S.L.) medium. Media were incubated at 36°C for up to two months.

Primary identification. Isolates were identified as MAC members by using the commercial AccuProbe MAC culture identification kit (Gen-Probe, CA) and by phenotypical characteristics: growth temperature at 25°C, 36°C, and 43°C; tellurite reduction, Tween 80 hydrolysis; nitrate reduction test; and sodium chloride tolerance (18).

PCR analysis. Bacterial cells were suspended in 200 μl of sterile water and boiled for 10 min; then they were centrifuged, and the supernatant was used as the DNA template for the PCRs. The identification of the isolates as *M. avium* or *M. intracellulare* was carried out by PCRs aimed at specific regions of the 16S rDNA (5, 43). PCR amplification of the insertion sequences IS*901* and IS*1245* was performed as described previously (13, 20). The PCR and sequencing of the *hsp65* gene were carried out as described previously in *M. avium* isolates (29, 35, 39).

For confirmation of the identity of the IS*1245*-negative *M. avium* isolates, 12 isolates were randomly selected (seven from urine samples and five from environmental sources) and subjected to PCR amplification and restriction enzyme analysis (REA) of IS*1311*; amplified DNA was digested with the restriction enzymes HinfI and MseI (New England Biolabs, Hitchin, Hertfordshire, United Kingdom) as described elsewhere (23). Analysis of the 16S rDNA and 16S-23S rDNA ITS sequences was performed in four isolates (two environmental cultures and two isolates from urine samples) (5, 31). PCR primers used in all reactions are described in Table 2. Positive and negative controls were included in each batch of tests.

PFGE analysis. *M. avium* subsp. *hominissuis* isolates were typed by PFGE analysis as described before (24). Briefly, bacterial cells were cast into low-melting-point agarose plugs and lysed with lysozyme (1 mg/ml), 1% sodium dodecyl sulfate, and proteinase K (1 mg/ml). Plugs containing DNA were digested with 20 U of XbaI for 4 h (New England Biolabs). The digested DNA was loaded in a 1% agarose gel and run in Tris-borate-EDTA buffer. PFGE was performed using a CHEF-DR III System (Bio-Rad Laboratories Ltd.) at 14°C for 20 h at 200 V. Gels were stained with ethidium bromide and photographed under UV light. The PFGE patterns obtained were interpreted as proposed by Tenover et al. (36); isolates were considered closely related if differences of no more than 2 to 3 bands were observed between the obtained PFGE profiles.

TABLE 2. Primers used in this study for the identification and characterization of the MAC isolates

Name or target	Direction[a]	Sequence (5′ to 3′)	Reference(s)
Mycgen-F/R	F	AGAGTTTGATCCTGGC TCAG	5, 43
	R	TGCACACAGGCCACAA GGGA	
Mycint	F	CCTTTAGGCGCATGTC TTTA	43
	R	TGCACACAGGCCACAA GGGA	
IS*901*	F	GCAACGGTTGTTGCTT GAAA	20
	R	TGATACGGCCGGAATC GCGT	
IS*1245*	F	GCCGCCGAAACGAT CTAC	13
	R	AGGTGGCGTCGAGG AAGA	
IS*1311*	F	GCGTGAGGCTCTGTGG TGAA	23
	R	ATGACGACCGCTTGGG AGAC	
ITS	F	TTGTACACACCGCCC GTCA	31
	R	TCTCGATGCCAAGGCATC CACC	
hsp65 3′ fragment	F	CGGTTCGACAAGGGTT ACAT	39
	R	ACGGACTCAGAAGTCC ATGC	
hsp65 5′ fragment[b]	F	ACCAACGATGGTGTGT CCAT	35
	R	CTTGTCGAACCGCATA CCCT	

[a] F, forward; R, reverse.
[b] Telenti fragment.

TABLE 3. Characterization of *M. avium* subsp. *hominissuis* isolates cultured from urine samples ($n = 23$) from 20 patients

Patient no.	Isolate reference	IS*1245*	*hsp65* sequevar	PFGE profile[a]	Infected with MAC[b]	Comment
1	MI05/02099	−	NA[c]	NA	No	
2	MI05/02102	−	Code 2	A*	No	
3	MI05/02103	−	Code 2	A	No	
4	MI05/03003	−	Code 2	A	No	HIV
5	MI05/03005	−	Code 2	A	No	
6	MI05/03006	−	Code 2	A	No	
7	MI05/03007	−	Code 2	A	No	HIV
8	MI05/03009	−	Code 2	A	No	
9	MI05/03010	−	Code 2	A	No	
10	MI05/03011	−	Code 2	A	No	
11	MI05/03012	−	Code 2	A*	No	HIV
12	MI05/03014	−	Code 2	A	No	
13	MI05/03015	−	Code 2	A	No	
14	MI05/03017	−	Code 2	A*	No	
15	MI06/00268	−	Code 2	A	No	
16	MI06/00269	−	Code 2	A	No	
17	MI05/02104	−	Code 2	A	No	
	MI05/03002	−	Code 2	A	No	
18	MI05/02100	−	NA	NA	No	
	MI05/02101	−	Code 2	A	No	
	MI06/00263	−	Code 2	A	No	
19	MI06/00266[d]	+	Code 1	B	Yes	HIV
20	MI06/00272	+	Code 1	Unrelated	Yes	HIV

[a] *, pattern closely related to PFGE pattern A.
[b] Based on clinical criteria.
[c] NA, not available.
[d] Patient with MAC isolates cultured from urine, sputum, and blood samples.

RESULTS

PCR results. All 64 isolates were identified as belonging to the *Mycobacterium* genus based on the results of the AccuProbe test and on the detection of specific regions of the 16S rDNA. Fifty-five isolates were identified as *M. avium*, and nine were identified as *M. intracellulare* by detection of a specific 16S rDNA fragment. The IS*901* PCR gave negative results in all 55 *M. avium* isolates under study. IS*1245* was detected in 11 *M. avium* isolates, all from clinical samples (Table 3, Table 4, and Table 5).

Clinical isolates. The 23 isolates cultured from urine samples from 20 patients were identified as belonging to MAC, but only two of them harbored IS*1245* (Table 3). Sequencing of the 3′ end of the *hsp65* gene was performed on 21 isolates, 19 IS*1245*-negative isolates, and the 2 IS*1245*-positive isolates. The code 2 *hsp65* sequevar (39), a typical sequevar of *M. avium* subsp. *hominissuis*, was obtained in all 19 isolates lacking IS*1245*. The two IS*1245*-positive isolates contained a code 1 sequevar. All of the IS*1245*-negative code 2 isolates were cultured from samples from patients who were later considered by clinical criteria not to be infected with MAC (Table 3). The two patients from whom the IS*1245*-positive isolates came were the only ones considered infected by MAC. In the first case, *M. avium* was also isolated from blood and sputum of the same patient; all three isolates shared the same code 1 *hsp65* sequevar, and the two PFGE-typed isolates shared the same profile. Thus, this patient was thought to have a disseminated infection. In the second case, the patient had a history of

TABLE 4. Characterization of *M. avium* subsp. *hominissuis* isolates cultured from sputum samples ($n = 11$) from 11 patients

Patient no.	Isolate reference	IS*1245*	*hsp65* sequevar	PFGE profile	Infected with MAC[b]	Comment
22	MI06/00271	+	Code 1	Unrelated	No	HIV
23	MI06/03008	+	Code 2	A	No	HIV
24	MI07/01955	−	Code 2	NA[a]	No	
25	MI07/01958	−	Code 2	NA	No	
26	MI07/01959	−	Code 2	NA	No	
27	MI07/01960	−	Code 2	NA	No	HIV
28	MI05/03018	−	Code 2	A	No	HIV
29	MI06/00273	+	Code 2	A	Yes	
30	MI07/01954	+	Code 9	NA	Yes	
33	MI06/00262	−	Code 15	Unrelated	Yes	
19	MI06/00267[c]	+	Code 1	B	Yes	

[a] NA, not available.
[b] Based on clinical criteria.
[c] Patient with MAC isolates cultured from urine, sputum, and blood samples.

TABLE 5. Characterization of *M. avium* subsp. *hominissuis* isolates cultured from sterile samples ($n = 4$) from three patients

Patient no.	Isolate reference	Sample	IS*1245*	*hsp65* sequevar	PFGE profile[a]	Infected with MAC[b]	Comment
37	MI06/00264	Liver biopsy	+	Code 3	Unrelated	Yes	HIV
39	MI05/03004	Blood	+	Code 1	Unrelated	Yes	HIV
19	MI06/00265[c]	Blood	+	Code 1	NA	Yes	HIV
	MI06/03278[c]	Blood	+	Code 1	NA	Yes	HIV

[a] NA, not available.
[b] Based on clinical criteria.
[c] Patient with MAC isolates cultured from urine, sputum, and blood samples.

repetitive *M. avium* isolations in urine samples obtained using a catheter; the strain isolated presented a code 1 in the *hsp65* analysis, and its PFGE profile was considered unrelated to all the others obtained in the study.

Nineteen MAC isolates were cultured from sputum samples of 17 patients. Eight isolates were identified as *M. intracellulare*, seven of which were causing disease in immunocompetent patients, while one isolate could not be associated with any pathology. The remaining 11 isolates were identified as *M. avium* (Table 4), and four of these isolates were associated with disease in the patients (three of these isolates harbored IS*1245* and contained different *hsp65* sequevars [codes 1, 2, and 9], and one was IS*1245* negative and contained a code 15 *hsp65* sequevar [GenBank accession no. EU085419]). Seven *M. avium* isolates came from patients not infected with MAC, and five were IS*1245* negative and contained a code 2 *hsp65* sequevar. The other two were IS*1245* positive and showed different *hsp65* codes (1 and 2).

The three isolates from blood samples, two from the same patient, contained the IS*1245* element and had the code 1 *hsp65* sequevar. The strain coming from a liver biopsy presented the IS*1245* element and had a code 3 *hsp65* sequevar (Table 5). Finally, the isolate from the bone marrow sample was identified as *M. intracellulare*. All of the isolates cultured from sterile samples were causing disease.

Environmental isolates. All of the 17 isolates were identified as *M. avium*. The IS*1245* element was absent in all of them. The sequencing of the *hsp65* 3′ end revealed the same sequevar (code 2) in 16 strains (no signal was obtained in 1 strain).

IS*1311* REA. In the 12 IS*1245*-negative *M. avium* isolates analyzed by means of the IS*1311* PCR and subsequent digestion with HinfI and MseI, the expected fragments for the *M. avium* 285- and 323-bp bands and 419- and 189-bp bands, respectively, were obtained; thus, this confirmed the identification of the IS*1245*-negative isolates as *M. avium* strains.

Four IS*1245*-negative isolates were also subjected to 16S rDNA and ITS sequencing, and identical sequences were obtained for the four strains in both fragments. The 16S rDNA sequence from the four isolates showed one single-nucleotide polymorphism (T instead of G at nucleotide 445) with the *M. avium* 104 strain sequence (GenBank accession CP000479). The ITS sequence obtained matched perfectly with the Mav-A sequevar described previously (6, 12).

PFGE results. Thirty-eight isolates yielded readable patterns after digestion with the XbaI restriction enzyme: 29 from clinical samples (21 from urine samples, 6 from sputum samples, and 1 from a blood and a liver biopsy sample) (Table 3, Table 4, and Table 5) and 9 from environmental samples. Nine unrelated patterns were observed. All of the environmental isolates, 19 out of 21 isolates from urine, and 3 out of 6 from sputum yielded identical (pattern A) or closely related (A*) patterns. All of these isolates also contained a code 2 *hsp65* sequevar and, with the exception of two isolates cultured from sputum samples, harbored no copies of IS*1245*. A second pattern (pattern B) was found in two isolates coming from sputum and urine samples from the same patient. The remaining isolates showed unrelated patterns.

DISCUSSION

This report describes the usefulness of several molecular techniques in the correct diagnosis and study of the epidemiology of MAC infections. Genetic tools, including commercial kits, for identification of MAC members are now available to mycobacteriology laboratories. Still, many clinical laboratories just identify the isolates as MAC members, as the treatment is the same for all MAC infections (4). Therefore, only incomplete epidemiological studies can be carried out. In our study, a combination of techniques was required to precisely identify the isolates to a subspecies level and to assess their relatedness.

IS*1245* is considered to be highly specific to *M. avium* (13), so it is commonly used for identification and typing (40). However, *M. avium* strains lacking IS*1245* were first described by Ritacco et al. (30) and were later reported as being isolated from human or animal sources (3, 19, 22); nonetheless, they were considered a rare finding and were always found in low proportions compared with the IS*1245*-positive strains. In most cases, characterization of IS*1245*-negative isolates is based only on commercial probes that might not identify the isolate unambiguously to the species-subspecies level. In our study, the identity of the *M. avium* strains lacking IS*1245* was confirmed by 16S rDNA, ITS, and *hsp65* sequencing and by IS*1311* REA and represented 81.8% of all *M. avium* isolates cultured, regardless of the source. The *hsp65* sequencing was a useful tool for confirmation of species identification of IS1245-negative *M. avium* isolates as previously described (4). Though PFGE and IS*1245* RFLP had been shown to have similar discriminative powers, PFGE is the only applicable typing technique for these isolates.

Molecular tests demonstrated that the same *M. avium* strain lacking IS*1245*, with a code 2 *hsp65* sequevar and a certain PFGE profile, pattern A, was isolated from all environmental samples, most of the urine samples (21 out of 23, or 91.3%), and several respiratory specimens (5 out of 19, or 26.3%).

The clinical significance of the isolation of MAC members from nonsterile human samples is a controversial issue. In this

way, MAC-positive culture has been shown to occur in respiratory specimens from patients without clinical disease (38); this could happen due to contamination of the sample or transient or persistent colonization. Likewise, results of direct observation of mycobacteria by acid-fast staining in urine samples should be interpreted carefully.

The molecular characterization correlated with clinical significance in that the 18 patients in whose urine the IS*1245*-negative code 2 *hsp65* sequevar was found were considered not infected by MAC by clinical criteria. On the other hand, the two *M. avium* isolates cultured from urine samples that harbored IS*1245* and had a code 1 *hsp65* sequevar came from two patients likely to have an *M. avium* infection: the first patient was thought to have a disseminated infection, and the second had a history of repetitive *M. avium* isolations in urine.

The explanation of the IS*1245*-negative code 2 *hsp65* sequevar isolations from certain urine samples could be attributed to the collection method: in this hospital, patients collect their urine samples into sterile receptacles, but intermediate containers might be used when the patient is handicapped. These intermediate containers are washed with tap water and soap after every use but not always sterilized while used by the same patient. The environmental isolates were cultured from the sediment obtained from the intermediate containers or from the taps of the patient's rooms. Thus, it is likely that urine samples collected using these containers would be contaminated by the environmental MAC strains. MAC members have been shown to grow in biofilms, increasing their resistance to disinfection methods such as chlorination (33); the most prevalent strain found in our study might persistently colonize the water supply system of the hospital as it was isolated from water samples from different floors of the hospital during a 3-year period.

The possibility of contamination of urine samples during the collecting process in the hospital is enhanced by the fact that, in samples collected in patient's houses, the recovery rate of *M. avium* is lower. In this context, in the period 1996 to 2003, 3,195 urine samples from admitted patients were cultured for the presence of mycobacteria, and 79 (2.47%) were positive. In the same period, 3,486 samples from outpatients were also collected and analyzed, and only 4 (0.11%) yielded positive isolation of MAC (E. Gomez-Mampaso, unpublished data).

Unlike the urine sample results, in the sputum samples the IS*1245*-negative code 2 *hsp65* sequevar strain represented only 5 out of the 19 MAC isolates recovered (again, they were considered as nonpathogenic). Sputum samples are collected directly into sterile receptacles, and therefore contamination of these samples is unlikely to occur; however, patients can be exposed to aerosols from shower and tap water from the bathrooms. If the water harbors *M. avium*, it could be a possible source of contamination.

Regarding *M. intracellulare*, eight isolates were recovered from respiratory samples and one from bone marrow. All except one (isolated from sputum) were found to be disease causing, highlighting the importance of this bacteria as a human pathogen.

Hospital water has been reported as a possible source of MAC infection (1, 38, 41). In these studies some degree of relationship was found between environmental *M. avium* and strains isolated from clinical samples (sputum, blood, or bone marrow). However, tap water was not usually added during the collection of the samples from which the cultures were recovered, and therefore contamination with environmental *M. avium* strains was considered unlikely. In our setting, we consider more possible a contamination of certain samples due to an incorrect collection method in some special cases of disabled patients. This hypothesis is supported by the fact that most of the patients (15 out of 20, or 75%) with positive isolations of MAC in urine were immunocompetent and were considered afterwards uninfected by MAC based on further medical analysis. Therefore, the environmental strain seemed to have low virulence since, even though it was widely distributed, it was not found to be pathogenic in any patient. However, the risk of infection by this strain cannot be ruled out, particularly in the case of HIV patients. To our knowledge, there are no studies that correlate the absence of IS*1245*, the *hsp65* sequevar, the PFGE pattern, and virulence, and therefore further research is needed to find out if these results can be extrapolated to other settings.

In summary, these results highlight the possibility of false-positive acid-fast staining or isolations of MAC members in nonsterile clinical samples, especially in the case of urine specimens. Therefore, it is important to characterize the environmental mycobacteria present in the facilities in order to exclude false-positive isolation in samples susceptible to contamination. For this purpose a combination of molecular techniques is required to correctly characterize MAC isolates. In our study we have demonstrated the presence of a particular strain persistently colonizing the water supply system and representing a potential source of contamination of samples and of infection in patients.

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