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Detection of Carbapenemase Genes in Aquatic Environments in Rio de Janeiro, Brazil

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This study reveals the presence of different carbapenemase genes (bla_{KPC}, bla_{NDM}, bla_{GES}, and $bla_{OXA48\text{-}like}$ genes) detected directly from water samples and clonal dispersion (by pulsed-field gel electrophoresis [PFGE] and multilocus sequence typing [MLST]) of KPC-2-producing *Enterobacteriaceae* in two important urban aquatic matrixes from Rio de Janeiro, Brazil, highlighting the role of aquatic environments as gene pools and the possibility of community spreading.

Until recently, the role of aquatic environments as antimicrobial resistance gene reservoirs has been overlooked. The contamination of natural aquatic environments with resistance-carrying bacteria and ingestion of this water during the practice of water sports could be an important route to spread resistance genes (1–3).

The purpose of this study was to investigate the presence of carbapenemase genes in the Rodrigo de Freitas Lagoon (RFL) and the Carioca River (CR), two important aquatic environments in Rio de Janeiro, Brazil. The RFL is an important landmark and an area for sports and fishing activity. The CR begins in an area of environmental preservation, runs underground through several neighborhoods of Rio de Janeiro City, and finally flows into Flamengo Beach on Guanabara Bay, where the presence of *Enterobacteriaceae* hosting bla_{KPC} has been previously reported (4).

Superficial water samples (2 liters) were collected according to American Public Health Association (APHA) methods (5) in March 2013 from 4 points in the RFL (RFL1 to RFL4) and in July 2013 from five points in the CR (CR1 to CR5) (Fig. 1). According to Brazilian standards (6, 7), all points from RFL water were considered unsuitable for bathing. At the first point of the CR (CR1), which is a well-preserved area, the water was considered acceptable and clear. However, along the river, the water quality decreased, suggesting the continuous discharge of sewage from nearby houses and hospitals (CR2 and CR3). In CR4 (immediately beyond the wastewater treatment plant [WWTP-FLOTFLUX]), the water was considered acceptable, and at CR5, the water quality decreased and was considered unacceptable because of its mixing with seawater from Guanabara Bay (see Table S1 in the supplemental material).

In order to select carbapenemase-producing isolates, 100 μl of concentrated water (centrifugation at 9,509 × *g* for 1 h at 4°C, twice) was plated on Mueller-Hinton agar, glutamate starch agar phenol red, and eosin-methylene blue agar (Oxoid) containing 2 mg/liter ceftazidime (Sigma-Aldrich) or 0.5 mg/liter ertapenem (Merck Sharp and Dohme). Different morphotypes of Gram-negative organism CFU were selected from each medium and submitted to the disk diffusion test for imipenem, meropenem, and ertapenem (Oxoid) (8, 9). The isolates exhibiting nonsusceptibility to at least one carbapenem were submitted for detection of bla_{KPC} and bla_{NDM} genes by conventional PCR and sequencing (10–12). The KPC-positive isolates were identified by matrix-assisted laser desorption ionization–time of flight (MALDI-TOF) mass spectrometry (Vitek MS; bioMérieux), and species of the *Enterobacter cloacae* complex were identified by sequencing of the *hsp60* gene (13).

A total of 242 Gram-negative bacillus isolates were recovered from the water samples, 132 from the RFL and 110 from the CR. By the disk diffusion method, 110 isolates from the RFL (83.3%) and 91 from the CR (82.7%) were considered nonsusceptible to at least one carbapenem tested (Table 1).

The bla_{KPC} and bla_{NDM} genes were not detected in isolates recovered from the RFL, but in 33 isolates (13.6%) from the CR, the $bla_{KPC\text{-}2}$ gene was detected (23 isolates from CR2, 6 from CR3, and 4 from CR5). Seven isolates were identified as *Klebsiella pneumoniae*, one was identified as *Klebsiella* sp., three were identified as *Aeromonas punctata*, one was identified as *Aeromonas hydrophila*, and 21 were identified as belonging to the *Enterobacter cloacae* complex: 12 classified as *Enterobacter kobei*, six classified as *Enterobacter asburiae*, and three classified as *E. cloacae* (Table 1).

Macrorestriction with XbaI digestion following pulsed-field gel electrophoresis (PFGE) was used to determine the genetic relatedness of the $bla_{KPC\text{-}2}$-positive isolates (14). Six clonal groups were found among the *K. pneumoniae* isolates, two were found among the *A. punctata* isolates, six were found among the *E. kobei* isolates, one was found among *E. asburiae* isolates, and two were

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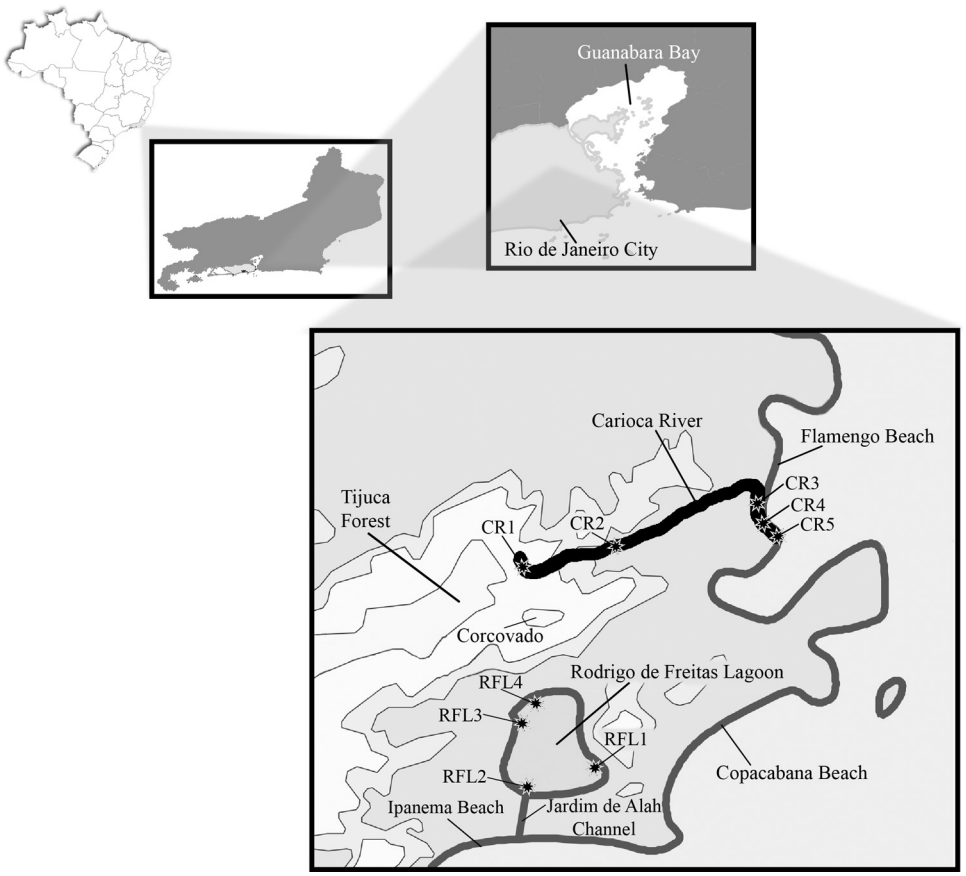

FIG 1 Rodrigo de Freitas Lagoon and Carioca River in Rio de Janeiro, Brazil. Stars show the points of water collection.

found among *E. cloacae* isolates. The clonal group EaA (*E. asburiae*) was found in CR2 and CR3, and clonal groups EkA and EkC (*E. kobei*) were detected in CR2 and CR5 (Table 2). The detection of the same clonal group of *E. kobei* and *E. asburiae* at different points of water collection shows the capacity of these clones to persist and carry their genes along the river, even after passage through the WWTP. The capacity of KPC-producing bacteria to pass through a hospital WWTP has also been shown elsewhere (15, 16).

By multilocus sequence typing (MLST) analysis, six sequence types (STs) were found among *K. pneumoniae* isolates (Table 2). Although we did not observe any of the STs previously described in Brazilian hospitals in our samples, one isolate belonged to ST1792 (clonal complex CC515), which is placed in the same evo-

TABLE 1 Bacterial isolates and carbapenemase genes recovered from each water collection point in Rodrigo de Freitas Lagoon and Carioca River, Rio de Janeiro City

Collection point	No. of isolates of carbapenemase-producing bacteria recovered			Carbapenemase gene(s) amplified from water sample (C_T)[b]
	Total	NS-Carba[a]	KPC producing (species)	
RFL1	28	25		16S
RFL2	39	30		16S/bla_{NDM} (25.53)
RFL3	27	24		16S[c]/bla_{KPC}[c] (32.86)
RFL4	38	34		16S/bla_{GES-16}
CR1	12	8		16S[c]
CR2	37	33	23 (*A. hydrophila*, 1; *A. punctata*, 2; *E. asburiae*, 2; *E. cloacae*, 3; *E. kobei*, 8; *K. pneumoniae*, 6; *Klebsiella* sp., 1)	16S/bla_{KPC} (24.08)/bla_{GES-16}/$bla_{OXA-48-like}$
CR3	23	21	6 (*A. punctata*, 1; *E. asburiae*, 4; *K. pneumoniae*, 1)	16S/bla_{KPC} (24.85)/bla_{GES-16}/$bla_{OXA-48-like}$
CR4	3	0		16S[c]/bla_{KPC}[c] (35.44)
CR5	35	29	4 (*E. kobei*, 4)	16S/bla_{KPC} (30.71)/bla_{GES-16}/$bla_{OXA-48-like}$

[a] Nonsusceptible to at least one of the carbapenems tested (ertapenem, imipenem, and meropenem).
[b] Cycle threshold determined by real-time PCR.
[c] Genes amplified only by real-time PCR.

TABLE 2 Molecular characteristics of KPC-2-producing isolates from the Carioca River, Rio de Janeiro, Brazil[a]

Collection point and identification	PFGE profile	ST	β-Lactamase(s)	Nonsusceptibility profile by disk diffusion test	MIC (μg/ml) by Etest				
					IPM	MEM	ETP	TGC	PMB
CR2									
A. hydrophila			KPC-2	CAZ, ATM, SXT, FOX, CTX, AMK, GEN	0.38	0.38	3	0.5	1
A. punctata	ApA		KPC-2, OXA-370, GES-31	FEP, CAZ, ATM, FOX, CTX, AMK	6	24	24	0.5	1.5
E. cloacae	EcA		KPC-2	FEP, CAZ, ATM, FOX, CTX	12	>32	>32	0.5	0.32
	EcB		KPC-2	FEP, CAZ, ATM, FOX, CTX	3	1.5	4	0.75	0.38
E. asburiae	EaA		KPC-2	FEP, ATM, FOX, CTX	4	3	4	1	1
E. kobei	EkA		KPC-2	ATM, FOX, CTX	2	2	1.5	0.38	0.38
	EkB		KPC-2	FEP, CAZ, ATM, FOX	6	>32	8	1	0.38
	EkC		KPC-2	CAZ, ATM, FOX, CTX	2	1.5	8	0.75	1
	EkD		KPC-2	CAZ, ATM, FOX, CTX	8	>32	>32	0.38	0.5
	EkE		KPC-2, GES-16	FEP, CAZ, ATM, FOX, CTX, GEN	6	12	32	0.75	1
K. pneumoniae	KpA	1792	KPC-2	ATM, FOX	1	0.75	1	0.75	0.5
	KpB	1791	KPC-2	ATM	1	1.5	8	0.75	0.38
	KpC	1245	KPC-2	FEP, CAZ, ATM, CTX	12	32	>32	0.75	0.5
	KpE	1793	KPC-2, GES-16	FEP, CAZ, ATM, SXT, FOX, CTX	12	12	8	1	0.38
	KpF	1794	KPC-2, GES-16	FEP, CAZ, ATM, CTX	1.5	1	3	0.5	0.38
Klebsiella sp.			KPC-2	FEP, CAZ, ATM, FOX, CTX	>32	>32	>32	0.125	0.5
CR3									
A. punctata	ApB		KPC-2	FEP, CAZ, ATM, FOX, CTX	3	4	16	0.5	0.38
E. asburiae	EaA		KPC-2	FEP, CAZ, ATM, FOX, CTX	2	3	>32	0.5	0.38
K. pneumoniae	KpD	1795	KPC-2	FEP, CAZ, ATM, CTX	4	24	16	0.75	0.5
CR5									
E. kobei	EkA		KPC-2	FEP, ATM, FOX, CTX	3	1	3	0.5	0.38
	EkC		KPC-2	FEP, CAZ, ATM, CTX	2	2	8	0.75	0.5
	EkF		KPC-2	FEP, CAZ, ATM, FOX, CTX	1.5	1	8	0.5	0.38

[a] Isolates without repeating of clonal group and sequence type that were recovered at the same point. Abbreviations: ST, sequence type; FEP, cefepime; CAZ, ceftazidime; ATM, aztreonam; SXT, trimethoprim-sulfamethoxazole; FOX, cefoxitin; CTX, cefotaxime; AMK, amikacin; GEN, gentamicin; IPM, imipenem; MEM, meropenem; ETP, ertapenem; TGC, tigecycline; PMB, polymyxin B.

lutionary branch as CC11 and CC258, both associated with the spread of the bla_{KPC} gene and previously described in Brazil (17). Furthermore, it is interesting that ST1245, found in one isolate, was first described in a KPC-2-producing isolate recovered from a WWTP in Austria (18).

By conventional PCR and amplicon sequencing (bla_{IMP}, bla_{VIM}, bla_{GES}, bla_{SPM}, $bla_{OXA-48-like}$, $bla_{OXA-23-like}$, $bla_{OXA-51-like}$, $bla_{OXA-24-like}$, $bla_{OXA-58-like}$, and $bla_{OXA-143}$) (19–23), we detected bla_{GES-16} in two KPC-2-producing *K. pneumoniae* isolates and in one *E. kobei* isolate from point CR2. In water from this point, we also detected an *A. punctata* isolate carrying $bla_{OXA-370}$ and a new $bla_{GES-like}$ variant named bla_{GES-31} (Table 2). The bla_{KPC} and bla_{GES-16} genes were previously reported in Brazilian recreational waters (4). However, although $bla_{OXA-370}$, an OXA-48-like family member, had already been described in hospitals in Rio de Janeiro, it had still not been detected in an aquatic environment (24).

According to antimicrobial susceptibility tests (Etest [AB Biodisk, Solna, Sweden] and the agar diffusion method [Oxoid]), many of the KPC-producing isolates from the CR were resistant only to β-lactams and presented MIC_{50}s lower than those normally found in clinical isolates (17) (Table 2).

These antimicrobial susceptibility profiles associated with the detection of bla_{KPC} in different clonal groups of different species, including environmental ones (*A. punctata* and *A. hydrophila*) and an isolate of *A. punctata* harboring bla_{KPC-2}, $bla_{OXA-370}$, and bla_{GES-31}, highlight the role of aquatic environments as a genetic library and disseminator of resistance genes.

In this way, we investigated the presence of carbapenemase genes directly from concentrated water samples by conventional PCR, sequencing as described above, and multiplex real-time PCR using Mastermix NAT (Bio-Manguinhos, Fiocruz) and primers and probes for bla_{NDM} (VIC), bla_{KPC} (6-carboxyfluorescein [FAM]), and 16S rRNA (CY5), performed on the Applied Biosystems 7500 real-time PCR system (Thermo Fisher Scientific) based on the CDC protocol (25). The amplification of the 16S rRNA gene was used to confirm the presence of bacterial DNA in water samples (26).

By conventional PCR and sequencing, bla_{NDM} and bla_{GES-16} could be amplified in RFL samples and bla_{KPC}, $bla_{OXA-48-like}$, and bla_{GES-16} could be amplified in CR samples. Real-time PCR could detect bla_{KPC} and bla_{NDM} from the same points as those found by conventional PCR, but it was also able to detect bla_{KPC} from points RFL3 and CR4. These results suggest that even the operating WWTP (CR4) is not sufficient to eliminate the carbapenemase genes (Table 1).

This study evidenced the dissemination of different carbapenemase genes (bla_{KPC}, bla_{NDM}, $bla_{OXA-370}$, and bla_{GES-16}) in two important aquatic environments in Rio de Janeiro, Brazil, highlighting the role of aquatic environments as gene pools and the

possibility of community spread. However, more studies are needed to determine the real risk to public health.

Nucleotide sequence accession number. The sequence for bla_{GES-31} has been deposited in GenBank under accession number KX034181.

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REFERENCES

1. **Leonard AF, Zhang L, Balfour AJ, Garside R, Gaze WH.** 2015. Human recreational exposure to antibiotic resistant bacteria in coastal bathing waters. Environ Int **82:**92–100. http://dx.doi.org/10.1016/j.envint.2015.02.013.
2. **Dufour AP, Evans O, Behymer TD, Cantu R.** 2006. Water ingestion during swimming activities in a pool: a pilot study. J Water Health **4:**425–430.
3. **Coleman BL, Salvadori MI, McGeer AJ, Sibley KA, Neumann NF, Bondy SJ, Gutmanis IA, McEwen SA, Lavoie M, Strong D, Johnson I, Jamieson FB, Louie M, ARO Water Study Group.** 2012. The role of drinking water in the transmission of antimicrobial-resistant *E. coli*. Epidemiol Infect **140:**633–642. http://dx.doi.org/10.1017/S0950268811001038.
4. **Montezzi LF, Campana EH, Correa LL, Justo LH, Paschoal RP, da Silva IL, Souza MDCM, Drolshagen M, Picao RC.** 2015. Occurrence of carbapenemase-producing bacteria in coastal recreational waters. Int J Antimicrob Agents **45:**174–177. http://dx.doi.org/10.1016/j.ijantimicag.2014.10.016.
5. **Rice EW, Baird RB, Eaton AD, Clesceri LS (ed).** 2012. Standard methods for the examination of water and wastewater, 22nd ed. American Public Health Association, Washington, DC.
6. **CONAMA.** 2005. Resolução N° 357, de 17 de março de 2005. CONAMA, Brasilia, Brazil. http://www.mma.gov.br/port/conama/res/res05/res357005.pdf.
7. **CONAMA.** 2000. Resolução N° 274, de 29 de novembro de 2000. CONAMA, Brasilia, Brazil. http://www.mma.gov.br/port/conama/res/res00/res27400.html.
8. **EUCAST.** 2015. Antimicrobial susceptibility testing. EUCAST disk diffusion method. http://www.eucast.org/fileadmin/src/media/PDFs/EUCAST_files/Disk_test_documents/Manual_v_5.0_EUCAST_Disk_Test.pdf.
9. **EUCAST.** 2015. Breakpoint tables for interpretation of MICs and zone diameters. http://www.eucast.org/fileadmin/src/media/PDFs/EUCAST_files/Breakpoint_tables/v_5.0_Breakpoint_Table_01.pdf.
10. **Naas T, Cuzon G, Villegas MV, Lartigue MF, Quinn JP, Nordmann P.** 2008. Genetic structures at the origin of acquisition of the beta-lactamase bla_{KPC} gene. Antimicrob Agents Chemother **52:**1257–1263. http://dx.doi.org/10.1128/AAC.01451-07.
11. **Nordmann P, Poirel L, Carrer A, Toleman MA, Walsh TR.** 2011. How to detect NDM-1 producers. J Clin Microbiol **49:**718–721. http://dx.doi.org/10.1128/JCM.01773-10.
12. **Otto TD, Vasconcellos EA, Gomes LH, Moreira AS, Degrave WM, Mendonca-Lima L, Alves-Ferreira M.** 2008. ChromaPipe: a pipeline for analysis, quality control and management for a DNA sequencing facility. Genet Mol Res **7:**861–871. http://dx.doi.org/10.4238/vol7-3X-Meeting04.
13. **Hoffmann H, Roggenkamp A.** 2003. Population genetics of the nomenspecies *Enterobacter cloacae*. Appl Environ Microbiol **69:**5306–5318. http://dx.doi.org/10.1128/AEM.69.9.5306-5318.2003.
14. **Ribot EM, Fair MA, Gautom R, Cameron DN, Hunter SB, Swaminathan B, Barrett TJ.** 2006. Standardization of pulsed-field gel electrophoresis protocols for the subtyping of *Escherichia coli* O157:H7, *Salmonella*, and *Shigella* for PulseNet. Foodborne Pathog Dis **3:**59–67. http://dx.doi.org/10.1089/fpd.2006.3.59.
15. **Picao RC, Cardoso JP, Campana EH, Nicoletti AG, Petrolini FV, Assis DM, Juliano L, Gales AC.** 2013. The route of antimicrobial resistance from the hospital effluent to the environment: focus on the occurrence of KPC-producing *Aeromonas* spp. and *Enterobacteriaceae* in sewage. Diagn Microbiol Infect Dis **76:**80–85. http://dx.doi.org/10.1016/j.diagmicrobio.2013.02.001.
16. **Chagas TP, Seki LM, da Silva DM, Asensi MD.** 2011. Occurrence of KPC-2-producing *Klebsiella pneumoniae* strains in hospital wastewater. J Hosp Infect **77:**281. http://dx.doi.org/10.1016/j.jhin.2010.10.008.
17. **Pereira PS, de Araujo CF, Seki LM, Zahner V, Carvalho-Assef AP, Asensi MD.** 2013. Update of the molecular epidemiology of KPC-2-producing *Klebsiella pneumoniae* in Brazil: spread of clonal complex 11 (ST11, ST437 and ST340). J Antimicrob Chemother **68:**312–316. http://dx.doi.org/10.1093/jac/dks396.
18. **Galler H, Feierl G, Petternel C, Reinthaler FF, Haas D, Grisold AJ, Luxner J, Zarfel G.** 2014. KPC-2 and OXA-48 carbapenemase-harbouring Enterobacteriaceae detected in an Austrian wastewater treatment plant. Clin Microbiol Infect **20:**O132–134. http://dx.doi.org/10.1111/1469-0691.12336.
19. **Poirel L, Le Thomas I, Naas T, Karim A, Nordmann P.** 2000. Biochemical sequence analyses of GES-1, a novel class A extended-spectrum beta-lactamase, and the class 1 integron In52 from *Klebsiella pneumoniae*. Antimicrob Agents Chemother **44:**622–632. http://dx.doi.org/10.1128/AAC.44.3.622-632.2000.
20. **Jeong SH, Bae IK, Park KO, An YJ, Sohn SG, Jang SJ, Sung KH, Yang KS, Lee K, Young D, Lee SH.** 2006. Outbreaks of imipenem-resistant *Acinetobacter baumannii* producing carbapenemases in Korea. J Microbiol **44:**423–431.
21. **Gales AC, Menezes LC, Silbert S, Sader HS.** 2003. Dissemination in distinct Brazilian regions of an epidemic carbapenem-resistant *Pseudomonas aeruginosa* producing SPM metallo-beta-lactamase. J Antimicrob Chemother **52:**699–702. http://dx.doi.org/10.1093/jac/dkg416.
22. **Higgins PG, Lehmann M, Seifert H.** 2010. Inclusion of OXA-143 primers in a multiplex polymerase chain reaction (PCR) for genes encoding prevalent OXA carbapenemases in *Acinetobacter* spp. Int J Antimicrob Agents **35:**305. http://dx.doi.org/10.1016/j.ijantimicag.2009.10.014.
23. **Woodford N, Ellington MJ, Coelho JM, Turton JF, Ward ME, Brown S, Amyes SG, Livermore DM.** 2006. Multiplex PCR for genes encoding prevalent OXA carbapenemases in *Acinetobacter* spp. Int J Antimicrob Agents **27:**351–353. http://dx.doi.org/10.1016/j.ijantimicag.2006.01.004.
24. **Pereira PS, Borghi M, de Araujo CF, Aires CA, Oliveira JC, Asensi MD, Carvalho-Assef AP.** 2015. Clonal dissemination of OXA-370-producing *Klebsiella pneumoniae* in Rio de Janeiro, Brazil. Antimicrob Agents Chemother **59:**4453–4456. http://dx.doi.org/10.1128/AAC.04243-14.
25. **Centers for Disease Control and Prevention.** 2011. Multiplex real-time PCR detection of *Klebsiella pneumoniae* carbapenemase (KPC) and New Delhi metallo-β-lactamase (NDM-1). Centers for Disease Control and Prevention, Atlanta, GA. http://www.cdc.gov/HAI/pdfs/labSettings/KPC-NDM-protocol-2011.pdf.
26. **Relman DA.** 1993. Universal bacterial 16S rDNA amplification and sequencing, p 489–495. *In* Persing DH, Smith TF, Tenover FC, White TJ (ed), Diagnostic molecular microbiology: principles and applications. American Society for Microbiology, Washington, DC.