Early diagnosis of infective endocarditis using a commercial multiplex PCR assay in Egypt

Nevin G. Al-Rachidi[a], Amany A. El-Kholy[a], Mervat G. Elanany[a], May S. Soliman[a], Reem M. Hassan[a], Dina M. Bassiouny[a], Hussien H. Rizk[b]

Departments of [a]Clinical Pathology, [b]Cardiology, Faculty of Medicine, Cairo University, Cairo, Egypt

Correspondence to Reem M. Hassan, MD, Clinical and Chemical Pathology Department, Faculty of Medicine, Cairo University, 1 Al-Saray St., Al-Manial, Cairo, 11562, Egypt; Tel: +20 114 139 9472; fax: +202 23649281; e-mail: reem.mostafa@kasralainy.edu.eg

Received 2 May 2016
Accepted 14 October 2016

Kasr Al Ainy Medical Journal
2017, 23:67–72

Context
Diagnosis of infective endocarditis (IE), particularly in blood culture-negative patients, has been a problem and requires further investigations.
Aim
This study was designed to evaluate the added value of a commercial multiplex PCR performed on blood in the early diagnosis of IE and compare its performance with conventional blood culture and serological testing, at a tertiary care center in Egypt.
Settings and design
Thirty-seven cases of diagnosed definite endocarditis were enrolled in the study.
Patients and methods
For each patient, blood culture was prepared and serum sample was obtained for serology testing for *Brucella* spp., *Bartonella* spp., *Coxiella burnetii* antibodies, and *Aspergillus* galactomannan antigen. Patients were selected for commercial Seegene multiplex PCR (sepsis screening) when their blood culture remained negative after 24 h incubation.
Statistical analysis
Sensitivity, specificity, positive predictive value, negative predictive value, and κ-test were used in statistical analysis for agreement.
Results
Thirty-seven cases were diagnosed as having definite IE. Causative organisms were detected using blood cultures in 18.9% (7/37) of cases after 24 h of incubation. Blood culture remained negative in 81% (30/37) of cases until the end of 21 days of incubation. One case showed positive result in serological testing for *Aspergillus* galactomannan Ag, whereas serological tests for *Bartonella* spp., *C. burnetii*, and *Brucella* spp. were negative in all cases. Multiplex PCR (sepsis screening) showed positive results in 51.3% (19/37) of definite IE cases.
Conclusion
The added value of multiplex PCR to conventional blood culture and the serological testing decreased the percentage of unidentified cases of IE from 81 to 48.7% in the selected study group.

Keywords:
blood culture, infective endocarditis, multiplex real-time PCR, serological testing

Kasr Al Ainy Med J 23:67–72

1687-4625

Introduction

Although most infective endocarditis (IE)-associated microorganisms are detected on cultures and occasionally with serology, diagnosis in the coming decade will likely rely more on molecular techniques to detect or to identify bacteria in patients with IE, as it has considerably improved the etiological diagnosis with accuracy, efficiency, and expected widespread availability. This is especially true in the case of culture-negative endocarditis following earlier antibiotic therapy or inability of fastidious micro-organisms to grow [1].

Several molecular assays have been used for the identification of microbial pathogens present in blood, including hybridization or amplification based methods. Broad-range prokaryotic PCR in conjunction with DNA sequencing of the amplicon and/or the use of specific probes is the PCR type commonly used and allows sufficient discrimination among different bacterial species. It has been applied to many types of samples for the diagnosis of different infections, including blood stream infections, neonatal sepsis, and IE [2,3].

Multiplex PCR assays are new approaches that can decrease the time to definite diagnosis of blood stream

 DOI: 10.4103/1687-4625.218997

infection (BSI) from days to hours [4,5], thus avoiding the dependence on only culture-based microbiological methods for diagnosis. There are several multiplex assays such as PCR/ES-MS, SeptiTest, and SeptiFast for the rapid detection of many microorganisms in 6–8 h, but some of them did not include drug resistance genes in their panel [6–9].

A commercial kit, the Magicplex Sepsis Test (Seegene, Seoul, Korea), appeared in the market in July 2010. It was designed to detect most pathogenic microorganisms responsible for BSIs along with three common drug resistance genes, *mecA*, *vanA*, and *vanB*, within 6 h. The assay includes two specific steps: a selective human cell lysis pretreatment of specimens to enrich for bacterial nucleic acids, and a dual-priming oligonucleotide that is used for amplification.

Dual-priming oligonucleotide consists of two functional priming regions separated by a poly (I) linker that generates dual-priming regions, resulting in only target-specific products. Those primers are labelled with a fluorescence marker that allows early detection of fluorescence signals through READ (real amplicon detection) technology and gives rapid results [10].

This study was designed to evaluate the impact of a commercial multiplex PCR (Seegene Magicplix TM Sepsis) performed on blood in the early diagnosis of IE after 24 h of incubation when blood culture is negative and compare its performance with conventional blood culture and serological testing at a tertiary care center in Egypt.

Patients and methods

Study design

The study included 37 patients diagnosed with IE referred to the Endocarditis Service, affiliated to the Cardiology Department, Cairo University Hospitals, from October 2013 to September 2014. Modified Duke's criteria were used for case definition [11]. All patients were subjected to history taking, including demographic data, presenting symptoms, underlying heart disease, comorbid conditions, and contact with animals. For each patient, a data collection sheet with epidemiologic, clinical, echocardiographic, and laboratory data was filled out. Informed consent was obtained from each patient before enrollment. The Clinical and Chemical Pathology Department's (Kasr Alainy) Ethical Committee approval was obtained for this study.

Laboratory methods

Blood cultures

At least three sets of blood culture were obtained as soon as possible after enrollment. We used the BACTEC Plus aerobic/F and BACTEC Plus anaerobic/F blood culture vials (Becton, Dickinson Sparks, MD, UAE). All bottles were incubated in BACTEC 9240 instrument for a total incubation period of 21 days, to be able to detect fastidious microorganisms that can cause IE. Positive bottles were then subcultured and colonies were identified according to standard Gram staining and biochemical reactions [12], and then antimicrobial susceptibility testing was carried out using the disc diffusion method (Mast Diagnostics, Mast Group Ltd, Merseyside, UK) and MIC determination using the E-test (bioMérieux Inc., Hazelwood, Missouri, USA), both according to CLSI 2014 recommendations [13].

Molecular techniques

Patients were selected for the study when their blood culture remained negative after 24-h incubation period. The samples used for blood culture and the Magicplex Sepsis Test were derived from the same venipuncture.

DNA extraction

One milliliter of fresh whole blood on EDTA tubes was transported to the laboratory and stored at 2–8°C until processing. The specimens were discarded if the test was not performed after 24 h. Microbial nucleic acids were enriched by performing a pretreatment with a blood pathogen kit (Cat. No. D-360–048; Seegene) according to the manufacturer's instructions, and then microbial DNA was extracted from pretreated samples and purified in the SEEPREP12 instrument (Seegene) according to the manufacturer's instructions. This extraction step requires nearly 2.5 h [14,15].

Multiplex real-time PCR using Seegene Magicplix sepsis

Microbial nucleic acids were amplified using a conventional PCR SEE AMP Seegene Thermoblock cycler (Seegene) in two independent reaction tubes: one for Gram-negative bacteria and fungi, and another for Gram-positive bacteria and three drug resistance markers: *mecA*, *vanA* and *vanB*; collectively, more than 90 pathogens known to cause blood stream infections were detected in this multiplex according to the manufacturer's instructions. This initial amplification process took ~2.5 h.

Amplified specimens were then detected and screened using CFX96 Real-time PCR System (Bio-Rad, Marnes-La-Coquette, France). Three real-time PCRs were performed: one for Gram-positive bacteria, one for drug resistance markers, and one

for Gram-negative bacteria and fungal pathogens. This step takes nearly 30 min.

Amplicons were detected using fluorescent nucleotide probes, and the results were analyzed using Seegene VIEWER real-time PCR software. An internal whole process control was used in all amplification steps, which is simultaneously amplified with target nucleic acids. To minimize contamination, DNA extraction and amplification/detection were performed in separated rooms.

Serologic testing

Agglutinating anti-*Brucella* antibodies were detected using the tube agglutination test (Linear Chemicals, Montgat-Barcelona, Spain). Testing for IgG antibodies against *Bartonella henselae* and *Bartonella quintana* and for IgG, IgM, and IgA antibodies against *Coxiella burnetii* was carried out using the indirect immunofluorescence assay (Vircell S.L. microbiologist, Granada, Spain). A patient was considered to have brucellosis when antibody titers for *Brucella* were at least 1/320, *Bartonella* endocarditis when IgG titers were at least 1 : 800, and *Coxiella* endocarditis when phase I IgG titer was at least 1 : 800 [11]. *Aspergillus* galactomannan antigen detection was carried out using the Platelia EIA (Bio-Rad). Patients with an index greater than 0.5 were considered positive for *Aspergillus* antigen [16].

Statistical methods

Statistical analysis was performed using SPSS statistical software version 15 (SPSS Inc., Chicago, Illinois, USA). Agreement between different diagnostic techniques was tested using κ-statistic. A P value of less than 0.05 was considered statistically significant.

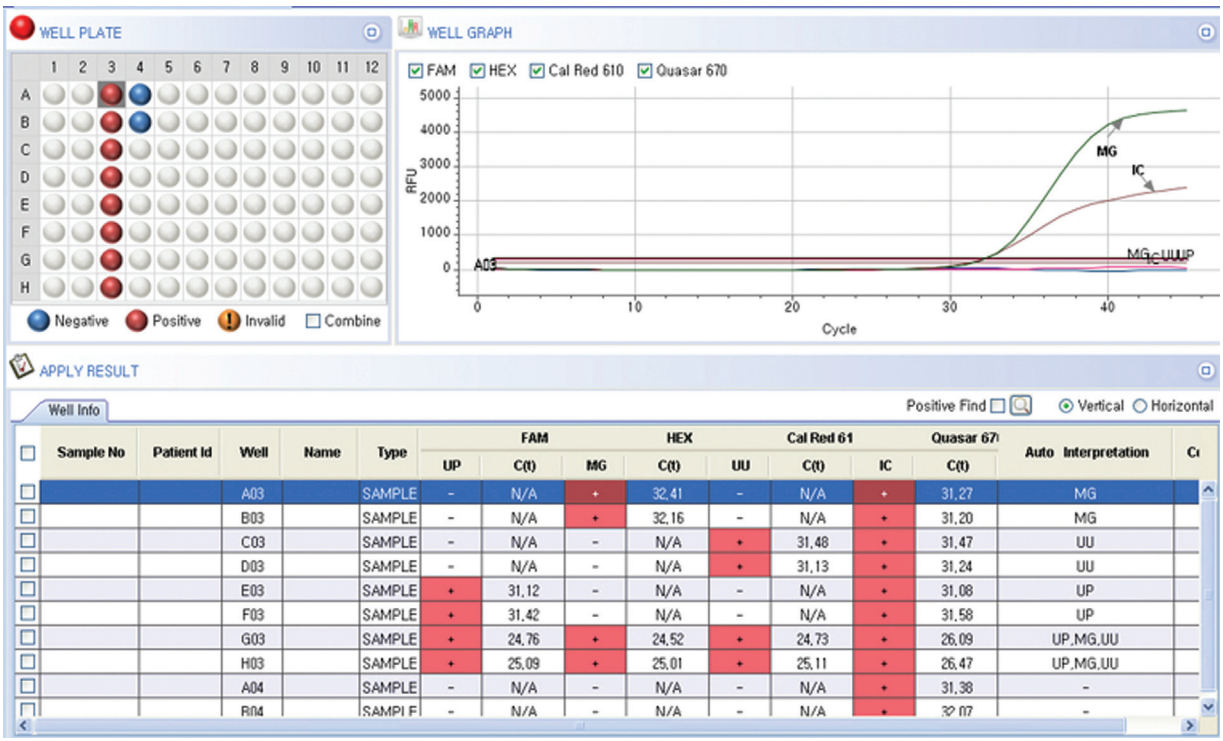
Results

Blood culture was positive in 18.9% (7/37) of definite cases of IE after 24 h of incubation. Blood culture positivity occurred after 3–6 days of incubation. *Staphylococcus aureus* (six cases) and *Pseudomonas* spp. (one case) were the organisms isolated from the culture-positive cases, with occurrence of methicillin resistance in five of six cases that grew *S. aureus*.

Of the 37 cases enrolled in our study, only one case showed positive result in serological testing for *Aspergillus* galactomannan Ag, whereas other serological tests for *Bartonella* spp., *C. burnetii*, and *Brucella* spp. showed negative results in all cases.

Multiplex PCR (sepsis screening) showed positive results in 51.3% (19/37) of definite IE cases and in 43.3% (13/30) of cases that remained blood culture-negative after 21 days of incubation but was negative in 48.7% (18/37) of definite IE cases. A representative PCR run of some cases is shown in Fig. 1.

Figure 1



Sample No	Patient Id	Well	Name	Type	FAM UP	FAM C(t)	FAM MG	HEX C(t)	HEX UU	Cal Red 61 C(t)	Cal Red 61 IC	Quasar 67 C(t)	Auto Interpretation	Cr
		A03		SAMPLE	-	N/A	+	32,41	-	N/A	+	31,27	MG	
		B03		SAMPLE	-	N/A	+	32,16	-	N/A	+	31,20	MG	
		C03		SAMPLE	-	N/A	-	N/A	+	31,48	+	31,47	UU	
		D03		SAMPLE	-	N/A	-	N/A	+	31,13	+	31,24	UU	
		E03		SAMPLE	+	31,12	-	N/A	-	N/A	+	31,08	UP	
		F03		SAMPLE	+	31,42	-	N/A	-	N/A	+	31,58	UP	
		G03		SAMPLE	+	24,76	+	24,52	+	24,73	+	26,09	UP,MG,UU	
		H03		SAMPLE	+	25,09	+	25,01	+	25,11	+	26,47	UP,MG,UU	
		A04		SAMPLE	-	N/A	-	N/A	-	N/A	+	31,38	-	
		B04		SAMPLE	-	N/A	-	N/A	-	N/A	+	32,07	-	

A representative PCR run of some cases (positive and negative) included in the study, analyzed on the Seegene VIEWER real-time PCR software.

Multiplex PCR evaluation against blood culture, regarded as the gold standard for the diagnosis of IE [11], showed a sensitivity of 85.7% [95% confidence interval (CI)=42.13–99.64%], specificity of 60.6% (95% CI=42.14–77.09%), positive predictive value of 31.6% (95% CI=12.58–56.55%), and negative predictive value of 95.2% (95% CI= 76.18–99.88%).

The relation between multiplex PCR and blood culture results showed an agreement of 50% in negative cases and of 15% in positive cases, collectively 65% between the two methods. Data are detailed in Tables 1 and 2.

Discussion

Many studies evaluated the use of multiplex PCR assays in the diagnosis of sepsis but few of them evaluated the added value of multiplex PCR assays for diagnosing IE. This specific patient population has unique criteria that make their definite diagnosis a big dilemma, especially in limited-resource settings. Blood culture-negative IE cases are high due to prior antibiotic intake, low bacterial load, and infection with fastidious microorganisms [1].

In the present study, multiplex PCR showed positive results in 43.3% (13/30) of cases that remained blood culture-negative after 21 days of incubation, providing great help in such cases to obtain a causative organism guiding the antibiotic regimen choice and whole management plan. Multiplex PCR and blood culture showed an overall agreement of 65%, whereas multiplex PCR showed an improved sensitivity over blood culture in definite IE cases by almost 25%, decreasing unidentified cases of IE from 81 to 48.7% in the selected study group.

These findings are comparable to other studies conducted in the past few years using commercially available multiplex PCR. Most of the studies evaluated their use in the diagnosis of suspected sepsis, such as the study conducted on 306 patients from emergency department who were subjected to blood culture and multiplex PCR. They found that blood culture was successful in the identification of more cases of septicemia compared with PCR among patients with an identified infectious etiology (66 and 46, respectively; P=0.0004), whereas PCR identified an additional 24 organisms that blood culture failed to detect. They concluded that real-time multiplex PCR increased the diagnostic yield, and thus shortened time to pathogen identification [8].

In the present study, multiplex PCR was positive in six of the seven blood culture-positive cases, missing only one case that showed *Pseudomonas* spp. on culture. Missing this case with PCR can be explained by the organisms included in the panel of Seegene sepsis

Table 1 The relation between blood Multiplex PCR and blood culture

			Blood culture		Total	*P* value
			Negative	Positive		
Blood Multiplex PCR	Negative	Count	20	1	21	
		% within Blood culture	60.6%	14.2%	52.5%	0.000*
		% of Total	50.0%	2.5%	52.5%	
	Positive	Count	13	6	19	
		% within Blood culture	39.3%	85.7%	47.5%	
		% of Total	32.5%	15.0%	47.5%	
Total		Count	33	7	40	
		% of Total	82.5%	7.5%	100.0%	

*P value <0.05 is considered significant.

Table 2 Comparison of organisms identified by blood culture and organisms identified by blood Multiplex PCR

Blood culture organism	Blood Multiplex PCR organism
Staphylococcus aureus (1)[a]	*Staphylococcus aureus* (1)[a]
Methicillin resistant *Staphylococcus aureus* (5)[a]	Methicillin resistant *Staphylococcus aureus* (5)[a]
Pseudomonas spp. (1)[a]	NR[b] (1)[a]
NR[b] (13)[a]	Methicillin resistant *Staphylococcus aureus* (6)[a], Enterococcus spp. (1)[a], Methicillin resistant *Staphylococcus aureus* and Enterococcus spp. (2)[a], Methicillin resistant *Staphylococcus aureus* and Gram negative Group B[c] (3)[a], Methicillin resistant

[a]Number of cases identified; [b]NR: negative result; [c]Gram negative Group B include: *Klebsiella pneumoniae*, *Klebsiella oxytoca*, *Proteus mirabilis*, *Escherichia coli*, *Enterobacter cloacae*, *Enterobacter aerogenes*.

screening, as it contains only *Pseudomonas aeruginosa* but other *Pseudomonas* spp. are not included.

This was also found in another study that compared the sensitivity of blood culture performed in 63 patients with the SeptiFast test and found that the SeptiFast was as sensitive as blood culture for organisms like *Streptococci*, *Enterococci*, and *S. aureus* (11/29 vs. 12/29). In contrast, SeptiFast could not detect coagulase-negative *Staphylococci* and other Gram-negative organisms detected on blood culture. However, three microorganisms were detected in IE patients treated with antibiotics, with blood culture negative on admission. They concluded that the SeptiFast test may be more useful if used with IE patients treated with antibiotics before admission [17].

Another study comparing the LightCycler SeptiFast real-time PCR with blood culture in IE patients had results similar to our study, in which concordant results between the two methods was obtained in 11/23 specimens (47.8%). They demonstrated that SeptiFast system has a good diagnostic performance and, when used concomitantly with blood culture, can improve sensitivity by up to 26%, especially with Gram-positive bacteria, in culture-negative IE patients [18].

The Seegene sepsis screening kit panel is restricted to certain organisms lacking some common causative agents of IE, such as *Bartonella* spp., *C. burnetii*, *Brucella* spp., *Legionella* spp., and the HACEK group (*Haemophilus* spp., *Actinobacillus actinomycetemcomitans*, *Capnocytophaga* spp., *Cardiobacterium hominis*, *Eikenella corrodens*, and *Kingella kingae*), which renders this test inadequate for diagnosing such cases.

Another study suggested the addition of a modified primer set in the kit panel of the multiplex real-time PCR SeptiFast (Roche Diagnostics, Basel, Switzerland) to improve the detection of microorganisms causing IE [19].

Moreover, the higher positivity of the results of the sepsis screen multiplex PCR, which may be due to its remarked sensitivity, necessitates strict adherence to aseptic precautions during sampling to avoid contamination with skin flora, and full identification of the positive cases to differentiate between pathogens and contaminants, using the identification kits. Nevertheless, the high yield may also be due to detection of remnants of DNA without the presence of viable organisms that could not be detected with blood culture because of prior antibiotic administration. However, this is a common drawback of all molecular-based techniques for the detection of microorganisms versus culture-based methods.

From the present study, we can establish the added value of molecular techniques in the setting of IE, especially culture-negative cases. However, PCR-based techniques cannot replace conventional culture techniques, especially in certain cases were micro-organisms are out of the multiplex panel. Multiplex PCR provides good help in rapidly detecting infectious causes in many cases of culture-negative endocarditis and time-saving for critically ill patients who cannot wait for blood culture results. Multiplex PCR requires only 6 h to provide results for micro-organism identification and common resistance patterns, thus guiding diagnosis and treatment regimen.

Financial support and sponsorship
Nil.

Conflicts of interest
There are no conflicts of interest.

References

1 Brouqui P, Raoult D. New insight into the diagnosis of fastidious bacterial endocarditis. FEMS Immunol Med Microbiol 2006; 47:1–13.

2 Klouche M, Schröder U. Rapid methods for diagnosis of bloodstream infections. Clin Chem Lab Med 2008; 46:888–908.

3 Hassan RM, El Enany MG, Rizk HH. Evaluation of broad-range 16S rRNA PCR for the diagnosis of bloodstream infections: two years of experience. J Infect Dev Ctries 2014; 8:1252–1258.

4 Paolucci M, Landini MP, Sambri V. Conventional and molecular techniques for the early diagnosis of bacteraemia. Int J Antimicrob Agents 2010; 36:6–16.

5 Reinhart K, Bauer M, Riedemann NC, Hartog CS. New approaches to sepsis: molecular diagnostics and biomarkers. Clin Microbiol Rev 2012; 25:609–634.

6 Bloos F, Sachse S, Kortgen A, Pletz MW, Lehmann M, Straube E, *et al.* Evaluation of a polymerase chain reaction assay for pathogen detection in septic patients under routine condition: an observational study. PLoS One 2012; 7:e46003.

7 Ecker DJ, Sampath R, Li H, Massire C, Matthews HE, Toleno D, *et al.* New technology for rapid molecular diagnosis of bloodstream infections. Expert Rev Mol Diagn 2010; 10:399–415.

8 Tsalik EL, Jones D, Nicholson B, Waring L, Liesenfeld O, Park LP, *et al.* Multiplex PCR to diagnose bloodstream infections in patients admitted from the emergency department with sepsis. J Clin Microbiol 2010; 48:26–33.

9 Wellinghausen N, Kochem AJ, Disqué C, Mühl H, Gebert S, Winter J, *et al.* Diagnosis of bacteremia in whole-blood samples by use of a commercial universal 16S rRNA gene-based PCR and sequence analysis. J Clin Microbiol 2009; 47:2759–2765.

10 Chun JY, Kim KJ, Hwang IT, Kim YJ, Lee DH, Lee IK, *et al.* Dual priming oligonucleotide system for the multiplex detection of respiratory viruses and SNP genotyping of CYP2C19 gene. Nucleic Acids Res 2007; 35:e40.

11 Li JS, Sexton DJ, Mick N, Nettles R, Fowler VG Jr, Ryan T, *et al.* Proposed modifications to the Duke criteria for the diagnosis of infective endocarditis. Clin Infect Dis 2000; 30:633–638.

12 Murray PR. Manual clinical microbiology. 9th ed. Washington, DC, USA: American Society for Microbiology ASM Press; 2011.

13 Clinical Laboratory Standards Institute. Performance standards for antimicrobial susceptibility testing. Twenty-third Informational Supplement M100-S23, 2014; Vol. 34, No. 1. CLSI Wayne, Pennsylvania, USA.

14 Handschur M, Karlic H, Hertel C, Pfeilstöcker M, Haslberger AG. Preanalytic removal of human DNA eliminates false signals in general 16S rDNA PCR monitoring of bacterial pathogens in blood. CIMID 2009; 32:207–219.

15 Hansen WL, Bruggeman CA, Wolffs PF. Evaluation of new pre-analysis sample treatment tools and DNA isolation protocols to improve bacterial pathogen detection in whole blood. J Clin Microbiol 2009; 47: 2629–2631.

16 Kalkhe AS, Rouphael N, El Chami MF, Workowski KA, Ganesh G, Jacob JT. Aspergillus endocarditis: a review of literature. Internat J Infect Dis 2010; 14:e1040–e1047.

17 Casalta JP, Gouriet F, Roux V, Thuny F, Habib G, Raoult D, *et al.* Evaluation of the LightCycler SeptiFast test in the rapid etiologic diagnostic of infectious endocarditis. Eur J Clin Microbiol Infect Dis 2009; 28:569–573.

18 Mencacci A, Leli C, Montagna P, Cardaccia A, Meucci M, Bietolini C, *et al.* Diagnosis of infective endocarditis: comparison of the LightCycler SeptiFast real-time PCR with blood culture. J Med Microbiol 2012; 61:881–883.

19 Conen A, Schaub N, Achermann Y, Battegay M, Frei R, Trampuz A. 19th European Congress of Clinical Microbiology and Infectious Diseases. Helsinki, Finland: Clin Microbiol Infect 2009; 15:107–612.