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## **New test for the diagnosis of bacterial endophthalmitis**

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Diagnosis, real-time PCR; bacterial endophthalmitis; infection

## **New test for the diagnosis of bacterial endophthalmitis**

### **Abstract**

**Background:** Diagnosis of bacterial endophthalmitis (BE) often fails due to: 1-reduced volumes of vitreous fluid (VF) and aqueous humor (AH); 2-lack of sensitivity of culture; 3-antibiotic treatments; 4-PCR cross-contamination and 5-limitations for real-time PCR melting curve interpretation. We developed a fast real-time-PCR to improve BE laboratory diagnosis performances.

**Methods:** a-PBS, VF, AH and cell suspensions spiked with *Bacteria*; b-VF and AH from endophthalmitis, and c-VF and AH from non-infective disorders were processed after adding an internal control (IC). DNA was extracted (MagnaPure®) and introduced into 4 tubes containing selected primers and probes for the identification and quantification of all *Bacteria* (Bac) and of 8 *Genera* by fast real-t PCR (f-real-t PCR). Performances of diagnosis based on direct microscopic examination, culture and f-real-t PCR were compared.

**Results:** The f-real-t PCR detected at least 0.01 CFU Bac/μl with no cross reactivity with fungi. Correlation with culture positive results was 100%. Sixty % of BE samples tested culture positive but f-real-t PCR tested positive for 90%. Samples from non-infective cases were negative.

**Conclusion:** The f-real-t PCR detects and quantifies *Bac*, *Staphylococci*, *Streptococci*, *Haemophilus*, *Pseudomonas*, *Enterobacteria*, *Acinetobacter*, *Propionibacteriaceae* and *Corynebacteria* in one run. Cultures required several hours to days (with non-negligible number of false negative results) and the f-real-t PCR was completed in 90 minutes. The f-real-t PCR appears as a new tool for the diagnosis of BE and its usefulness requires validation with larger series of samples.

## **Introduction**

Bacterial endophthalmitis (BE) is a sight threatening intraocular inflammation that may follow surgery, trauma and bacterial keratitis or may be of endogenous origin.

Ideally, all cases of BE would be culture proven but more than 30% of vitreous fluid (VF) and aqueous humor (AH) obtained from patients with endophthalmitis are culture negative.[1-3] The reduced volume of samples (VF 200 to 400  $\mu$ l; AH 50 to 150  $\mu$ l), the slowly or fastidious growing organisms, the low bacterial loads in ophthalmic samples and the antibiotherapy started before sampling reduce the diagnosis capacities of methods based on culture. In addition culture are biased because several *Bacteria* (Bac) can be cultivated only when their optimal metabolic requirements are reproduced *in vitro*.[2-9]

Immunoassays have been developed to improve culture performances, but results could be obtained only for samples containing over  $10^4$  CFU/ ml. False negative conclusions were frequently generated because as many as 45% of meningitis (and endophthalmitis) evolve with bacterial loads inferior to the detection threshold.[1-2, 10-11]

The nucleic-acid amplification based tests (NAAT) are less affected by the prior use of antibiotics and by slow growing species.[12-13]. Moreover, microorganisms have been detected by NAAT (PCR) in delayed onset of postoperative BE in samples testing culture negative.[2,3,6-9,14] However, numerous cross-contaminations were repeatedly reported for PCR.

The SYBR Green real-time technology is based on the production of signals that are detected during the nucleic-acid amplification process in reaction tubes kept closed. However, the complexity of the analysis and interpretation of the signals limited its use for BE diagnosis.[3, 5-9,15-9]

To improve bacterial diagnosis we conceived a new test based on the real-time PCR technology for which primers and molecular probes were selected. The molecular probes are able to trigger signals for which the relevance is automatically interpreted. Specimens spiked with bacterial suspensions and AH and VF from subjects with suspected intra ocular

infections were tested to assess the detection capacities of this test [load of all *Bacteria* (Bac) and *Genera* isolated most frequently from eye fluids].

## **Materials and methods**

### **Samples**

Investigations were performed in accordance with “Declaration of Helsinki” ([http:// www.wma.net /e/policy/](http://www.wma.net/e/policy/)). A total of 200 to 400 µl of VF or 30 to 150 µl of AH were obtained in the surgical ward (not diluted) for immunological, cytological and microbiological investigations. Aliquots were transferred into sterile DNA free microfuge coded tubes and stored at –80°C for f-real-t PCR. Control specimens consisted of VF and AH from non-infective patients.

### **Microbiological diagnosis**

Cytospin smears of VF and AH were stained by Giemsa’s pH: 7.4 and Gram’s methods for microscopic examination.

### **Culture**

The specimens were processed within 30 minutes after collection following routine validated diagnosis procedures [1-8, 15, 17-19]. Inoculated media were kept up to 30 days before they were discarded.

### **Preparation of quantified bacterial suspensions for sensitivity assessment**

To reduce the over representation of DNA from no viable microorganisms, one colony of *Staphylococci*, *Streptococci*, *Propionibacteriaceae*, *Corynebacteria*, *Enterobacteria*, *Pseudomonas*, *Haemophilus*, *Acinetobacter* and fungi was scraped from the agar plates, suspended and re plated on a solid rich agar. After 24 hours, one colony was scraped from the second plate, suspended in Phosphate Buffer Solution (PBS) and tenfold diluted. Each dilution was plated to quantify the bacterial load (CFU/µl).

## DNA extraction

The DNA was extracted from 100 µl using the MagNA Pure<sup>®</sup> Nucleic Acid isolation kit and the MagNA Pure<sup>®</sup> Compact equipment (Roche, France) and eluted in 100 µl, in a vertical safety laminar flow cabinet in a dedicated room. The preparation of reagents and the fast real-time PCR (f-real-t PCR) were carried out in separated areas. The internal control (IC) consisting in 5 µl of seal herpes virus (gift from G. J. van Doornum, Erasmus MC, Rotterdam, The Netherlands) was added before extraction to monitor the extraction yields and the absence of PCR inhibitors.[20-21]

## Primers and Probes

Primers were chosen from regions of identity within the 16S rDNA following the alignment of sequences outlined in Bergey's Manual of Determinative Bacteriology. [4] The BactUn probe was adapted from sequences reported for blood derivative control tests.[12,13] The sequences of primers and detection probes for Gram + cocci (G+ C), *Staphylococcus* (Staph), *Streptococci* (Strep), *Propionibacteriaceae* (Prop), *Corynebacteria* (Cory), *Pseudomonas* (Pse), *Haemophilus* (Hae) and *Acinetobacter* (Aci) were adapted from previous reports.[12,22] However, for *Corynebacteria* (Cory) and *Propionibacteriaceae* (Prop) original sets of primers and probes were designed [23] and validated with the primer Express software (Applied Biosystems, France). These original sequences are: Propi Primer-1: 5'ATACGTAGGGTGCGAGCGTTGTCC; Primer-2: 5'TGGTGTTCTCCTATATCTGCG C; Hybridization probe Propi: [AminoC6 +JOE] GATCGCGTCGGAAGTGTAATCTTGGG G [Black Hole Quencher (BHQ1)]. For Cory: Primer-1: 5'ATACGTAGGGTGCGAGCG TTGTCC; Primer-2: 5'TGGTGTTCTCCTGATATCTGCGC; Hybridization probe Cory: [Cy3]TCGCGTCGTCTGTGAAATCCCGGGG [BHQ2]. For the detection and quantification of the seal herpes virus the sequences are: Primer-1: GGGCGAATCACAGATTGAATC; Primer 2: GCGGTTCCAAACGTACCAA; and Hybridization probe [TET] TTTTATGTGT CCGCCACCATCTGGATC [TAMRA].[20-21] If more than one probe had to be introduced into one reaction tube, the differences in the emission wavelength spectrums of the fluorophores were of at least 15 nanometers.

## PCR conditions

Testing was carried out by adding 10 µl of the extracted DNA to each of the 4 tubes containing the primers (0.5µM) and fluorophore-labelled TaqMan® probes (0.5µM) in 10 µl of TaqMan® FAST Universal PCR Mastermix (Applied Biosystems-France Ref. 4352042). The cycling program consisted in 1 cycle at 95°C for 10 min and 45 cycles of amplification (15 s at 95°C, 8 s at 52°C, and 10 s at 72°C). Each run contained negative controls with no template and DNA extracts from the reactants.

### Confirmation of negativity and testing for viable *Bac*

The absence of viable *Bac* was confirmed by dividing the samples immediately after arrival at the laboratory in 2 tubes containing DNA-free culture broth (dilution 1/10). The first was kept for 4 h at 4°C and the second at 37°C. The absence of viable *Bac* (negativity) was confirmed if the Cts were similar for both aliquots and the presence of viable *Bac* was highly suspected if differences were >1.5 Cts (amplification cycles).

### Preparation of kits

Kits (stable at -20°C for at least 12 weeks) consist of 4 tubes containing 10 µl of FAST Master-MIX (Applied Biosystems, France)

Tube Number	Primer 1	Primer 2	Type of fluorophore and probe names	Detection and semi quantification of
1	Bact 1 <sup>(I;II)</sup>	Bact 2 <sup>(I;II)</sup>	a-FAM staph <sup>(I)</sup> b-Cy3 strep <sup>(I)</sup> c-6-JOE pseudo <sup>(I)</sup>	<i>Staphylococci</i> <i>Streptococci</i> <i>Pseudomonas</i>
2	Bact 1 <sup>(I;II)</sup>	Bact 2 <sup>(I;II)</sup>	a-FAM entero <sup>(I)</sup> b-Alexa hae <sup>(I)</sup> c-Cy3 aci <sup>(I)</sup> d-JOE grampos <sup>(III)</sup>	<i>Enterobacteria</i> <i>Haemophilus</i> <i>Acinetobacter</i> Gram + Cocci
3	BactUn1 <sup>(IV)</sup> PhHv1 <sup>(V)</sup>	BactUn2 <sup>(IV)</sup> PhHv2 <sup>(V)</sup>	Cy55 eubact <sup>(IV)</sup> TET IC <sup>(V)</sup>	Bac ( <i>eubacteria</i> ) Seal herpes (IC)
4	Propiocoryne1	Propiocoryne2	a-6-JOE propi b-Cy3 cory	<i>Propionibacteriaceae</i> <i>Corynebacteria</i>

Detailed sequences: I: [22]; II: [12]; III: [9, 22, 24]; IV: [13]; V: [20-21]

## Results

The bacterial loads were calculated from curves (Ct versus equivalent CFU/ $\mu$ l) for each bacterial suspension diluted in Phosphate Buffer Solution (PBS) before DNA extraction. Table I shows that universal and specific sets of primers and probes introduced in the 4 tubes produced signals for the 8 different *Genera* of Bac (*Genera*) at loads of 0.01 CFU/ $\mu$ l or lower. The f real-t PCR made possible to identify and quantify different *Genera* in water, saline, PBS, AH, VF and human cell suspensions. The Ct values -results reproducible for all the experiments- obtained for samples tenfold diluted, were delayed in 3 Cts / log CFU. This validated the capacity for bacterial quantification.

In Table II are presented the results obtained after testing 8 different *Genera*. The IC Ct values are reproducible, indicating that the DNA extraction procedures did eliminate the PCR inhibitors independently from the specimen in which Bac were suspended. All the species were positive, and the DNA extracted from *Staphylococci* and *Streptococci* were the only producing G+C signals. The DNA extracted from *S. pneumoniae* was detected in the eubact, G+C and strep channels and the DNA extracted from *Acinetobacteria* and *Pseudomonas* were positive for eubact but negative for *Enterobacteria*, confirming the specificity of the probes.

The global analysis of the results obtained with the f-real-t PCR shows no cross reactions between any of the bacterial or fungal strains (specificity of 100%). For all the *Genera* the detection limits (relative sensitivity) were of at least the equivalent 0.01 CFU/ $\mu$ l.

*Escherichia coli* (E.coli) and *Serratia marcescens* (S.marc) were detected by eubact and ente, and *Neisseria meningitidis*, *Neisseria gonorrhoea* and *Bacillus* by eubact, at loads of  $\leq$  0.01 CFU/ $\mu$ l. Indeed, this new f-real-t PCR was designed to detect all *Bac* but to identify the most frequently *Genera* associated to BE; However, the approach used for the design of this test allows the addition of primers and probes if is required the identification of additional *Genera*, species or genotypes.

The comparison of f-real-t PCR results with those obtained by classic techniques are presented in Table III. The microscopic examination reveals in VF and AH from clinically suspected endophthalmitis the presence of PMN (polymorphonuclear leukocytes) in 19 out of 20 samples (in 2 samples only 1 PMN was observed).



The f-real-t PCR was positive in 19 out of 20 samples; Prop was detected in 4 VF and in 4 AH, and Staph in 1 VF and in 4 AH. Culture was negative but real-t PCR positive in 9 samples presenting PMN. Cultures for *P.acnes* were obtained after at least > 70 h. Globally, cultures were positive in 65% and f-real-t PCR in 90%. However, none of the culture positive intraocular fluids was f-real-time PCR negative and none of the controls testing f-real-time PCR negative were positive for direct microscopic examination or culture. In 1 VF culture was positive for *Aspergillus* and in 1 AH culture was positive and for *C. albicans*. The controls carried out systematically with the reactants and with samples from the dedicated working areas were negative and the sterile unpreserved eye drop bottles, and the AH and VF from patients with signs not related to endophthalmitis tested f-real-t negative (100% specificity).

Gram staining did not detect *Bac* in 2 out of 3 eye drop bottles kept open overnight but the 3 were positive both for real-t-PCR and culture (results obtained after 72-96 hours). The G+ rods observed after Gram staining in 1 sample were confirmed 36 hours after by culture (*Bacillus sp*). For this sample the f-real-t PCR detected eubact signals. (approx 0.01 CFU/μl).

The VF and AH from uveitis, vitrectomy, posterior segment surgeries with non-infectious signs tested culture negative and f-real-t PCR negative (1 PMN was observed in 1 AH and lymphocytes (ly) in 2; ly were observed in 1 out of 5 VF).

Considering the clinical diagnosis of endophthalmitis as the gold standard for the diagnosis of endophthalmitis, the detection of more than 10 polymorphonuclear cells on the slides after cytocentrifugation of undiluted samples and Giemsa staining showed a relative sensitivity of 85% (17 positive out of 20). Gram staining detected *Bac* in 10 out of 20 clinical samples (relative sensitivity: 50%) and allowed the rapid detection of a filamentous fungi in one sample. Sensitivity of culture was 65% and results could be obtained after incubations periods of 24 or more hours in 14 out of 15 clinical samples (one sample with *Pseudomonas* was positive after 18 hours). Cultures were positive for fungi in 2 endophthalmitis samples (10%).

The f-real-t PCR was positive in 19 out of 20 samples (relative sensitivity of 95%). In one sample testing culture positive for yeast the f real-t-PCR detected *Bac* and the eubact result was confirmed by signals for *Enterobacteria*, suggesting a poly microbial infection.

**Table I: Detection capacities of the f-real-t PCR on PBS spiked samples #**

Detection system for #	Eq CFU/ $\mu$ l	Results##		Detection limit
		Ct BactUn probe	Ct for the specific probe	
<i>Staphylococci sp</i>	0.1	36.3	33.9	
	0.01	39.5*	37.2*	$\leq 0.01$ Eq CFU/ $\mu$ l of sample **
	0.001	40.9*	40.1	
<i>Streptococci sp</i>	0.1	35.1	34.7	
	0.01	38.2*	37.4*	$\leq 0.01$ Eq CFU/ $\mu$ l of sample **
	0.001	40.9*	40.2	
Gram + cocci	0.1	34.9	34.1	
	0.01	37.0	36.9	
	0.001	39.8*	39.5*	$\leq 0.001$ Eq CFU/ $\mu$ l of sample **
<i>Enterobacteria</i>	0.1	36.6	32.1	
	0.01	39.3*	35.2*	$\leq 0.01$ Eq CFU/ $\mu$ l of sample **
	0.001	40.0*	40.5	
<i>Haemophilus sp.</i>	0.0	34.1	34.0	
	0.01	37.2	37.0	
	0.001	39.7*	39.9*	$\leq 0.001$ Eq CFU/ $\mu$ l of sample **
<i>Acinetobacter sp.</i>	0.1	31.9	36.8	
	0.01	35.2	40.0*	$\leq 0.01$ Eq CFU/ $\mu$ l of sample **
	0.001	38.6*	42.5	
<i>Propionibacteriaceae</i>	0.1	36.0	33.0	
	0.01	39.2*	36.0*	$\leq 0.01$ Eq CFU/ $\mu$ l of sample **
	0.001	40.4*	>42	
<i>Pseudomonas sp</i>	0.1	34.4	34.0	
	0.01	37.5	36.9	
	0.001	38.9*	39.8*	$\leq 0.001$ Eq CFU/ $\mu$ l of sample **
<i>Corynebacteria</i>	0.1	35.8	33.8	
	0.01	39.0*	36.9*	$\leq 0.01$ Eq CFU/ $\mu$ l of sample **
	0.001	40.3*	39.9*	
<i>Bacillus sp.</i>	0.1	33.5	ND	
	0.01	36.4	ND	
	0.001	39.9*	ND	$\leq 0.01$ Eq CFU/ $\mu$ l of sample **
<i>Stenotrophomonas maltophilia</i>	0.1	36.1	ND	
	0.01	39.1*	ND	$\leq 0.01$ Eq CFU/ $\mu$ l of sample **
	0.001	42.1	ND	
<i>Streptococcus pneumoniae</i>	0.1	34.1	34.6 ###	
	0.01	37.0	37.5 *###	$\leq 0.01$ Eq CFU/ $\mu$ l of sample **
	0.001	39.8*	40.2 ###	

#: each quantified strain was suspended in PBS before DNA extraction; ##: Ct values for the ICs were ranged between 32.3 and 33.4; \*: Positivity was confirmed by the analysis of the second derivative. \*\*: Detection capacities were identical for *Bac* suspended 1/10 in PBS or in distilled water. ND: not determined; ###: Signal for strep.

**Table II Detection capacities of the f-real-t PCR on fluids spiked with *Bac* or fungi**

sample suspended (1/10) in					
sample Number		Distilled Water *	VF	AH	Human fibroblast suspensions (10 <sup>6</sup> / ml)
	1	Ct BactUn	36.9	37.6	36.7
Conclusion		+eubact; G+C; Staph	+eubact; G+C; Staph	+ eubact; G+C; Staph	+eubact; G+C; Staph
2	Ct BactUn	38.6	38.1	38.7	38.7
	Conclusion	+eubact; G+C; Staph	+eubact; G+C; Staph	+eubact; G+C; Staph	+eubact; G+C; Staph
3	Ct BactUn	37.9	38.6	37.1	38.0
	Conclusion	+eubact; G+C; Staph	+eubact; G+C; Staph	+eubact; G+C; Staph	+eubact; G+C; Staph
4	Ct BactUn	37.1	38.0	37.0	36.7
	Conclusion	+eubact; G+C; Staph	+eubact; G+C; Staph	+eubact; G+C; Staph	+eubact; G+C; Staph
5	Ct BactUn	35.9	36.1	36.3	36.7
	Conclusion	+eubact; G+C; Strep	+eubact; G+C; Strep	+eubact; G+C; Strep	+eubact; G+C; Strep
6	Ct BactUn	36.4	36.9	36.1	36.5
	Conclusion	+eubact; G+C; Strep	+ eubact; G+C; Strep	+eubact; G+C; Strep	+eubact; G+C; Strep
7	Ct BactUn	36.1	36.2	36.8	36.7
	Conclusion	+eubact; G+C; Strep	+eubact; G+C; Strep	+ eubact; G+C; Strep	+ eubact; G+C; Strep
8	Ct BactUn	36.1	36.9	36.1	36.2
	Conclusion	+eubact; Hae	+eubact; Hae	+ eubact; Hae	+eubact; Hae
9	Ct BactUn	37.9	36.9	37.7	37.4
	Conclusion	+eubact; Hae	+eubact; Hae	+eubact; Hae	+eubact; Hae
10	Ct BactUn	38.0	37.5	37.2	37.9
	Conclusion	+eubact; Hae	+eubact; Hae	+eubact; Hae	+eubact; Hae
11	Ct BactUn	37.2	37.1	37.0	37.3
	Conclusion	+eubact; Hae	+eubact; Hae	+ eubact; Hae	+ eubact; Hae
12	Ct BactUn	36.0	36.9	36.4	36.1
	Conclusion	+eubact; Cory	+eubact; Cory	+eubact; Cory	eubact; Cory
13	Ct BactUn	37.2	37.1	37.8	37.4
	Conclusion	+eubact; Ente	+eubact; Ente	+ eubact; Ente	+ eubact; Ente
14	Ct BactUn	37.1	37.8	37.2	37.9
	Conclusion	+eubact; Acine	+eubact; Acine	+eubact; Acine	+eubact; Acine
15	Ct BactUn	37.9	37.9	37.6	37.2
	Conclusion	+eubact; Ente	+ eubact; Ente	+ eubact; Ente	+ eubact; Ente
16	Ct BactUn	37.6	37.1	37.1	38.0
	Conclusion	+ eubact	+ eubact	+ eubact	+ eubact
17	Ct BactUn	37.4	37.0	37.0	37.3
	Conclusion	+ eubact	+ eubact	+ eubact	+ eubact
18	Ct BactUn	37.4	37.9	37.0	36.9
	Conclusion	+eubact; Pse	+eubact; Pse	+eubact; Pse	+eubact; Pse
19	Ct BactUn	37.5	37.9	37.5	37.5
	Conclusion	+ eubact	+ eubact	+ eubact	+ eubact
20	Ct BactUn	37.7	37.9	37.1	37.2
	Conclusion	+ eubact; Propi	+eubact; Propi	+eubact; Propi	+eubact; Propi
21	Ct BactUn	> 42	> 42	> 42	> 42
	Conclusion	all signals -	all signals -	all signals -	all signals -
22	Ct BactUn	> 42	> 42	> 42	> 42
	Conclusion	all signals -	all signals -	all signals -	all signals -
23	Ct BactUn	> 42	> 42	> 42	> 42
	Conclusion	all signals -	all signals -	all signals -	all signals -
24	Ct BactUn	> 42	> 42	> 42	> 42

	Conclusion	all signals -	all signals -	all signals -	all signals -
25	Ct BactUn	> 42	> 42	> 42	> 42
	Conclusion	all signals -	all signals -	all signals -	all signals -
26	Ct BactUn	> 42	> 42	> 42	> 42
	Conclusion	all signals -	all signals -	all signals -	all signals -
27	Ct BactUn	> 42	> 42	> 42	> 42
	Conclusion	all signals -	all signals -	all signals -	all signals -
28	Ct BactUn	> 42	> 42	> 42	> 42
	Conclusion	all signals -	all signals -	all signals -	all signals -
29	Ct BactUn	> 42	> 42	> 42	> 42
	Conclusion	all signals -	all signals -	all signals -	all signals -
30	Ct BactUn	> 42	> 42	> 42	> 42
	Conclusion	all signals -	all signals -	all signals -	all signals -
31	Ct BactUn	> 42	> 42	> 42	> 42
	Conclusion	all signals -	all signals -	all signals -	all signals -
32	Ct BactUn	> 42	> 42	> 42	> 42
	Conclusion	all signals -	all signals -	all signals -	all signals -

\*: Distilled water for injections; VF: vitreous fluid; AH: aqueous humor; Ct: number of cycles triggering specific positive signals. Conclusion: +eubact: detection of Bac in the sample; +G+C: Gram-positive cocci; +Staph: f *Staphylococci*; +Strep: detection of *Streptococci*; +Ente: *Enterobacteria*; Hae: *Haemophilus*; Pse: *Pseudomonas*; Aci: *Acinetobacter*; Propi: *Propionibacteriaceae*; Cory: *Corynebacteria*. \*\*: All the tubes containing the Mix 3 produced TET signals (PhHV) with Ct values ranged between 31.8 and 32.9. Sample numbers: 1: *Staph. epidermidis*; 2: Methicillin-sensitive *Staph. aureus*; 3: *Staph. haemolyticus*; 4: Methicillin-resistant *Staph. aureus*; 5: *Strep. mitis*; 6: *Strep. pneumoniae*; 7: *Strep. pyogenes*; 8: *Hae. influenzae type 1*; 9: *Hae. influenzae type 2*; 10: *Hae. influenzae type 3*; 11: *Hae. parainflueanzae type 1*; 12: *Corynebacteria*; 13: *Serratia marcescens*; 14: *Acinetobacter baumannii*; 15: *Escherichia coli*; 16: *Neisseria meningitidis*; 17: *Neisseria gonorrhoeae*; 18: *Pseudomonas aeruginosa*; 19: *Stenotrophomonas maltophilia*; 20: *Propionibacterium acnes*; 21: *Candida albicans*; 22: *Candida glabrata*; 23: *Candida lusitanae*; 24: *Candida tropicalis*; 25: *Candida krusei*; 26: *Cedosporium sp.*; 27: *Curvularia sp.*; 28: *Paecilomyces sp.*; 29: *Aspergillus niger*; 30: *Phoma sp.*; 31: Distilled water; 32: PBS

**Table III Comparison of classic bacterial diagnosis methods with the f-real- t PCR**

	Diagnosis test							
	Microscopic examination		Culture		f-real-t PCR			
	Cytology (PMN or ly)	Gram staining	Results	Time for results (hours)	Signal for Eubacteria (BactUn)	Specific signals		
G+C						Ente	Others	
Type of sample	<b>Results</b>							
VF clinical signs of endophthalmitis	PMN > 10	+CG+	stre A	28	+	+	-	stre
	PMN: 1	+CG+	stre B	28	+	+	-	stre
	PMN > 10	N	pse	18	+	-	-	pse
	PMN > 10	+fungi	Aspergillus	48	-	-	-	-
	PMN > 10	+G+R	pro	72	+	-	-	pro
	PMN > 10	N	N	-	+	+	-	pro
	PMN > 10	N	pro	72	+	-	-	pro
	PMN > 10	N	N	-	+	+	-	sta
	PMN > 10	N	N	-	+	-	-	pro
	not visible	N	pro	72	+	-	-	pro
Artificial tears (unpreserved eye drop bottles)	N	N	N	-	-	-	-	-
	N	N	N	-	-	-	-	-
	N	N	N	-	-	-	-	-
	* N	N	sta.N.coag	96	+	+	-	sta
	* N	+G+R	<i>Bacillus sp.</i>	36	+	-	-	ND
	* N	N	sta.N.coag	72	+	+	-	sta
AH clinical signs of endophthalmitis	PMN >10	N	N	-	+	-	-	pro
	PMN > 10	N	sta.N.coag	48	+	+	-	sta
	PMN > 10	+G+C	sta.N.coag	96	+	+	-	sta
	PMN > 10	N	N	-	+	-	-	pro
	PMN: 1	N	pro	144	+	-	-	pro
	PMN > 10	+G+C	sta.N.coag	24	+	+	-	sta
	PMN > 10	+G+C	pro	72	+	-	-	pro
	PMN > 10	+G+C	sta.N.coag	48	+	+	-	sta
	PMN > 10	N	yeast	96	+	-	-	ente
	PMN > 10	+G+C	sta.N.coag	48	+	+	-	sta
control VF (no clinical signs of endophthalmitis)	not visible	N	N	-	-	-	-	-
	not visible	N	N	-	-	-	-	-
	ly	N	N	-	-	-	-	-
	not visible	N	N	-	-	-	-	-
	not visible	N	N	-	-	-	-	-
control AH (no clinical signs of endophthalmitis)	not visible	N	N	-	-	-	-	-
	PMN: 1	N	N	-	-	-	-	-
	ly	N	N	-	-	-	-	-
	not visible	N	N	-	-	-	-	-
	ly	N	N	-	-	-	-	-

PMN >10: more than 10 polymorphonuclear cells/ field (400X); ly: lymphocytes (>5/field)\*: unpreserved eye-drop bottles were kept open for 24 h at 20°C.  
 +G+C: detection of Gram positive cocci; +G+R: detection of Gram positive rods; +sta: *Staphylococci*; + ente: *Enterobacteria*; +stre: *Streptococci*; +pro: *Propionibacteriaceae*; sta.N.coag: *Staph. Negative-coagulase*; S.epi: *Staph. epidermidis*.

## Discussion

A new ultra-rapid test was developed for the diagnosis of BE. This technique is able to simultaneously detect and quantify 8 different *Genera* of *Bac* and assess the DNA extraction yields and the presence of PCR inhibitors.

The culture methods are still considered as the “gold standard”. However they may produce false negative results due to the small VF and AH sample volumes, the sequestration of bacteria on solid surfaces (e.g. intraocular lens, lens remnants, and capsule), the use of antibiotics prior to sampling and the fastidious nature of *Bac*[1-7] Nevertheless, NAAT (PCR) are independent of the growth of the microorganisms and they are able to detect low amounts of injured and fastidious microorganisms and even those suspended in antibiotics.[12-15]

Different PCRs have been described for diagnosis of endophthalmitis, and all required post-amplification probe hybridization and/or sequencing. The combination of PCR with probe hybridization yielded positive results in 91 % of samples when Gram staining and culture only in 56%.[24] Nested-PCR (second run based amplification techniques) were also reported for BE diagnosis, but these tests required additional restriction fragment length polymorphism analysis and/or DNA sequencing, for which conclusions could be drafted after at least 3.5 h after the first PCR run.[25]

The rate of positive results obtained by culture of AH and VF was dramatically increased by mean of techniques amplifying the eubacterial genes conserved at species, genus, and kingdom level highly.[3, 6, 9,18, 25] Reports using the eubacterial gene amplification showed an increase in detection rates from 18% to 62% in post cataract endophthalmitis [18,25], and multicentric studies showed that eubact PCR increases *Bac* detection by at least 20% compared to Gram-staining and culture.[6] However, the eubacterial PCR only detects *Bac* and is unable to identify *Genera* (additional procedures like amplicon sequencing should be therefore carried out) enhancing the risks for DNA carryover. [8, 9, 16, 24-25]

Diagnosis techniques based on the SYBR Green real-time PCR were developed to reduce the risks for cross contamination because the amplification/detection diagnosis procedures are carried out in closed tubes and followed by melting-curve signal analysis. However, the

following reasons limited the use of this strategy for clinical diagnosis of BE: a- the SYBR Green binds to non-relevant traces of any kind of double-stranded DNA that may be present in the sample; b- identical DNA sequences can be found in different *Bac*; c- the *Bac* presenting melting profiles similar to species in reference databases are misidentified; d- the environmental DNA *Bac* traces leads to false positive conclusions, and e- the interpretation of results obtained for samples containing more than one *Bac* are difficult and not reliable. To overcome these limitations, additional procedures targeting internal fragments were described (nested PCRs), but these strategies increase the risks for amplified material dispersion. [3, 6, 24, 25]

The specificity of the TaqMan approach, compared to other NAAT is increased due to the supplementary fluorescent probe recognition required by the targeted bacterial sequence during each amplification step. In addition, the polymerase triggers the emission of fluorescent signals detected only at a pre selected wavelength.

This study shows that the whole f-real-t PCR procedure could be carried out in closed tubes without interferences of fluorescent signals that could have been triggered by traces of DNA from solvents, extraction reactants and environment.

## **Conclusions**

The new f-real-t PCR is able to detect and quantify in saline, PBS, AH, VF and cell suspensions all *Bac* and 8 *Genera* at levels of at least 0.01 Eq PFU/ $\mu$ l with a specificity of 100% and a sensitivity of 90% (endophthalmitis samples).

The results obtained in the present study suggest that the ideal ultra-rapid diagnosis tool for endophthalmitis should also include fungal detection (10% of the samples). However, the f-real-t PCR shows its potential for infections that would in all practicality remain underestimated by culture or for samples obtained after antibiotherapy onset, enabling rapid decisions within the limited time experienced in sight-threatening situations. The time for positive results could be reduced to 90 minutes using inexpensive *in-house* prepared kits that allow automatic diagnosis in only one amplification-detection run. The stringent procedures for sampling and the f-real-t PCR (tubes kept closed during the whole procedure) minimize

the potential risks for false positive conclusions, increasing operators' confidence. Multicentric trials should be carried out before conclusions on its usefulness for routine diagnosis can be drafted.

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