Novel diagnosis of fungal endophthalmitis by broad-range real-time PCR detection of fungal 28S ribosomal DNA

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Abstract
Aim To detect the fungal genome in the ocular fluids of patients with fungal endophthalmitis by using a novel broad-range polymerase chain reaction (PCR) system.
Methods After informed consent was obtained, ocular fluid samples (aqueous humor or vitreous fluids) were collected from 497 patients (76 patients with infectious endophthalmitis including clinically suspected bacterial and fungal endophthalmitis and 421 patients with infectious or non-infectious uveitis). Forty ocular samples from non-infectious patients without ocular inflammation were collected as controls. Fungal ribosomal DNA (28 S rDNA) was measured by a quantitative real-time PCR assay.
Results Fungal 28 S rDNA of the major fungal species, such as Candida, Aspergillus, and Cryptococcus, were detected by novel broad-range real-time PCR examination (>10^3 copies/ml). Fungal 28 S rDNA was detected in the ocular fluids of 11 patients with endophthalmitis or uveitis (11/497, 2.2%). All 11 positive samples were detected in the infectious endophthalmitis patients (11/76, 14.5%). These PCR-positive ocular fluids had high copy numbers of fungal 28 S rDNA (range, 1.7 × 10^3 to 7.9 × 10^6 copies/ml), which indicated the presence of fungal infection. Of the 11 patients who were PCR positive, further examinations led to a diagnosis of fungal endophthalmitis in ten patients. The fungal 28 S rDNA was detected in one non-infectious case (a false-positive case). In addition, there were two PCR false-negative cases that were clinically suspected of having fungal endophthalmitis.
Conclusions This novel quantitative broad-range PCR of fungal 28 S rDNA is a useful tool for diagnosing endophthalmitis related to fungal infections.

Keywords Polymerase chain reaction · Fungi · Ocular fluids · Endophthalmitis

Introduction
Fungal endophthalmitis can be caused by endogenous infections. These infections occur in patients who have systemic disorders (e.g., diabetes or malignancy), patients who use systemic drugs (e.g., broad-spectrum antibiotics, chemotherapeutic agents, or steroids), and patients who have intravascular catheters. In addition, fungal endophthalmitis can be caused by exogenous infections that arise from trauma or intraocular surgery. The clinical findings in some ocular infectious diseases caused by fungal species are quite diverse, with the exception of Candida infection. Candida infection in the eye is always characterized as endogenous endophthalmitis with fungal ball vitreous opacities. Moreover, fungal infections have been widely associated with various ocular disorders including endophthalmitis. Because of this diversity, it is often difficult to diagnose ocular fungal infections. Polymerase chain reaction (PCR) has been used to provide evidence of fungal involvement in suspected cases of intraocular infections. Previous studies have used PCR to demonstrate the presence of...
fungal DNA in the ocular fluids of patients with infectious endophthalmitis [1–3].

PCR-based methods make it possible to establish a diagnosis in less time than is required by standard cultures [4–6]. Moreover, studies have found that fungal cultures are negative in half of PCR-positive cases [1–3, 7]. The sensitivity of conventional culture techniques is not high, and these cultures take a long time due to their slow growth. Thus, the use of broad-range real-time PCR to analyze ocular samples may be a better way to obtain a rapid diagnosis in patients with unknown intraocular infectious diseases.

For the diagnosis of infectious endophthalmitis, broad-range real-time PCR for fungi is now available [1–3, 7]. To detect many types of fungal DNA, primers and probes for conserved regions in fungal sequences are used. We previously designed pan-fungal primers and probes that were complementary to the 18 S rRNA sequences present in the Candida and Aspergillus species, and we reported the efficacy of the technique for diagnosis [7]. This PCR technique detected all species of Candida and Aspergillus DNA. Although there were many advantages to using this PCR technique to diagnose fungal infection, there was one disadvantage. Although the fungal 18 S broad-range PCR detected Candida and Aspergillus DNA, it cannot detect other types of fungal DNA. Recently, a novel broad-range real-time PCR technique was developed for the rapid detection of human pathogenic fungi [8]. The assay targeted a part of the 28 S large subunit rRNA genes (28 S rDNA). Therefore, we prepared a new assay that targets a part of the 28 S rDNA found in species such as Candida, Aspergillus, Cryptococcus, Trichophyton, Mucor, Penicillium, and Pichia.

In the present study, we attempted to develop a novel fungal PCR examination that uses 28 S rDNA primers and the corresponding probes for the diagnosis of endophthalmitis related to fungal infection.

Materials and methods

Subjects

Based upon medical history and clinical observations, 497 patients (260 men and 237 women) were consecutively enrolled in a prospective study that was conducted at the Tokyo Medical and Dental University Hospital. The patient group consisted of patients with infectious endophthalmitis including clinically suspected bacterial and fungal endophthalmitis (n=76) and patients with infectious or noninfectious uveitis (n=421). The average patient age (±SD) was 60 (±16) years. After obtaining informed consent, samples of aqueous humor and vitreous fluids were collected from all patients.

In addition to the patient group, we also analyzed samples from a control group in which no patients had any type of ocular inflammation. The control patients were enrolled in this prospective study in 2009. Forty samples (20 aqueous humor samples and 20 vitreous fluid samples) were collected from the 40 control patients. The control group consisted of patients who had age-related cataract (n=20), macular edema (n=14), retinal detachment (n=4), idiopathic macular hole (n=1), and idiopathic epiretinal membrane (n=1).

For aseptic ocular sampling, the following procedures were performed in all subjects, as described in our previous reports [7, 9]. A 0.1-ml aliquot of aqueous humor was collected aseptically in a syringe with a 30-G needle. Half of the sample was then transferred into a pre-sterilized microfuge tube and used for PCR. In patients who were undergoing vitreous surgery, uncontaminated non-diluted vitreous fluid samples (0.5–1.0 ml) were collected during the diagnostic pars plana vitrectomy [7, 9]. Topical antibiotics were used in almost all patients before collecting samples, but oral antibiotics were not used.

The research followed the tenets of the Declaration of Helsinki, and all study protocols were approved by the Institutional Ethics Committee of Tokyo Medical and Dental University. The clinical trial was registered, and the information is available at www.umin.ac.jp/ctr/index/htm with study number R000002708. The study was started in July 2009 and was terminated in February 2011.

Quantitative polymerase chain reaction

DNA was extracted from the samples using a DNA Mini Kit (Qiagen, Valencia, CA, USA) installed on a Robotic workstation that was set for automated purification of nucleic acids (BioRobot EZ1 Advanced, Qiagen). The real-time PCR was performed by using an Amplitaq Gold and Light Cycler 480 II (Roche, Basel, Switzerland). Primers and probes of fungal 28 S rDNA are described elsewhere [8]. The sense primer was 5'-gcatacaataacgggagaaag-3', and the antisense primer was 5'-tagctttagtgatRaRtttacacc-3'. The probe (Dual-Labeled probe, Integrated DNA Technologies, Coralville, IA, USA) was 5'-FAM- cgccgatgaacgg- SaaRagctc–iowaBK-3'. Products were subjected to 50 cycles of PCR amplification, with cycling conditions set at 95 °C for 10 min, followed by 50 cycles at 95 °C for 0 s and 60 °C for 20 s. For PCR assay sensitivity, PCR fragments were amplified from the DNA of C. albicans (strain: ATCC 60193), A. flavus (strain: ATCC 22246), and C. neoformans (strain: ATCC 14116). The PCR results were obtained within 3 h after sample collection.
Amplification of the human β-globulin gene served as an internal positive extraction and amplification control. Fungal copy number values of more than 100 copies/ml in the sample were considered to be significant.

**Results**

Sensitivity of broad-range real-time PCR assay for fungal 28 S rDNA

To confirm the broad-range real-time PCR assay sensitivity, PCR fragments were amplified from the DNA of *Candida, Aspergillus*, and *Cryptococcus* species. The detection limit and standard range of the TaqMan real-time PCR were determined by using serial tenfold dilutions of linearized plasmid. The PCR results for the prepared samples showed that *C. albicans* DNA was detected at concentrations between 10² and 10⁵ copies/ml (Fig. 1A). In addition, *Aspergillus* (Fig. 1B) and *Cryptococcus* DNA (Fig. 1C) were also detected at concentrations between 10² and 10⁵ copies/ml. The best sensitivity for detecting *Candida, Aspergillus*, or *Cryptococcus* DNA was at a concentration of 10¹ copies/ml. No DNA was detected in the negative control (nuclease-free water).

Detection of fungal 28 S rDNA in suspected fungal endophthalmitis patients

The PCR results indicated that fungal 28 S rDNA was positive in 11 samples of ocular fluid from the endophthalmitis or uveitis patients (11/497, 2.2%). All 11 positive samples were detected in the infectious endophthalmitis patients (11/76, 14.5%). A representative PCR result in a case of endogenous endophthalmitis related to *Aspergillus* infection is shown in Fig. 2.

The PCR-positive patients had high copy numbers of fungal 28 S rDNA ranging from $1.7 \times 10^3$ to $7.9 \times 10^6$ copies/ml, which indicated the presence of fungal infection. Further examinations revealed that 10 of the 11 PCR-positive patients had fungal endophthalmitis; seven patients were diagnosed with endogenous endophthalmitis (*Candida*, *Aspergillus*, *Cryptococcus*, and 2 unknown), and three patients were diagnosed with late postoperative endophthalmitis (*Candida* and 1 unknown) (Table 1). Fungal 28 S rDNA was detected in only one non-infectious case (case 455 in Table 1). This PCR false-positive case had primary intraocular lymphoma that was diagnosed by monoclonal detection of B-cell IgH rearrangement by PCR, high amounts of IL-10 by ELISA, and detection of typical lymphoma cells (Class V) in the vitreous sample. Thus, fungal 28 S rDNA was detected in ocular samples from 10 patients with fungal endophthalmitis and one patient with non-infectious primary intraocular lymphoma.

However, two of the PCR-negative patients were clinically suspected to have fungal endophthalmitis (cases 24 and 461 in Table 1). PCR did not detect the fungal genome in the aqueous humor of these patients (<100 copies). *C. albicans* was detected in blood samples from case 461. Case 24 was a patient with endogenous endophthalmitis, and his blood tests were positive for β-D-glucan. Systemic antifungal and topical antifungal therapies were effective in the treatment of these two patients with false-negative results.

In conventional fungal cultures of ocular fluids, six (60%) of the 10 PCR-positive samples from fungal endophthalmitis patients were positive, and four samples were negative (Table 1). In addition, patients with fungicemia (cases 24, 179, 231, 326, 359, 461, and 490) had already begun therapy with antifungal agents before PCR examinations (Table 2). Among these seven patients, six patients received intravenous hyperalimentation. On the other hand, patients without clinically apparent fungicemia (cases 30, 77, 161, and 355) initiated antifungal drug therapy after receiving positive PCR results (Table 2).

The diagnostic parameters of sensitivity, specificity, positive predictive value, and negative predictive value of the PCR examinations for the diagnosis of fungal endophthalmitis were calculated to be 0.833, 0.998, 0.909, and 0.996, respectively.

**Discussion**

The broad-range real-time PCR assay amplified fungal 28 S rDNA in the ocular fluids of patients with clinically suspected fungal endophthalmitis. The 28 S rDNA of major fungal species for endophthalmitis, such as *Candida, Aspergillus*, and *Cryptococcus*, were detected by a novel broad-range real-time PCR examination (>10⁴ copies/ml). Our fungal endophthalmitis patients were all immunocompetent, but almost all patients were older than 60 years of age, with the exception of two patients (cases 268 and 490). The PCR examination was negative for fungal DNA in cases of ocular inflammation caused by bacterial endophthalmitis or uveitis. In addition, fungal DNA was not detected in any of the 40 control patients without ocular inflammation.

Broad-range PCR for the 28 S rRNA sequence proved to be a reliable tool for the diagnosis of fungal endophthalmitis. Moreover, real-time quantitative PCR can be used to determine whether or not the fungus is related to the endophthalmitis. By using this PCR system, we were able to rapidly diagnose various types of fungal endophthalmitis in a few
Fig. 1 PCR assay sensitivity. To examine the sensitivity of the broad-range real-time PCR for fungal 28S rDNA, PCR fragments were amplified from DNA of Candida (C. albicans, a), Aspergillus (A. flavus, b), and Cryptococcus (C. neoformans, c) species. The number in the parenthesis indicates the cycle threshold (Ct) value in quantitative PCR.
patients exhibiting clinical evidence of a fungal infection. In 40% of the PCR-positive patients, the fungal cultures that were performed on the same ocular fluid sample were negative. Thus, PCR-based methods make it possible to establish an etiologic diagnosis in less time than is required by standard cultures.

In addition, since these methods can detect very small numbers of DNA copies, they are extremely sensitive. These methods are also beneficial when used for intraocular infections, as only a relatively small volume of sample needs to be obtained at any one time. Since it is essential that treatments be started early in cases of infectious endophthalmitis, this broad-range real-time PCR system for ocular samples can provide a rapid diagnosis for patients who have an unknown intraocular disorder such as idiopathic uveitis or endophthalmitis. Additionally, when minimal amounts of ocular samples are available, it is difficult to perform a culture test to detect fungi [4–6]. Therefore, the use of PCR to detect the fungal genome in ocular fluids is advantageous.

We previously developed a novel PCR assay to detect fungal infection by amplifying fungal 18 S rRNA genes [7]. The broad-range real-time PCR detected a few Candida species (C. albicans, C. parapsilosis, C. tropicalis, C. guilliermondii, C. glabrata, and C. krusei), along with Aspergillus species (A. fumigatus, A. flavus, A. nidulans, A. niger, and A. terreus). By using several different primers and probes, we were able to separately detect each of these fungal species. Candida or Aspergillus DNA was detected in seven of 54 ocular samples (13%) from patients with unknown uveitis or endophthalmitis. These PCR-positive samples showed significantly high copy numbers of Candida or Aspergillus DNA. On the other hand, fungal DNA was not detected in the other 46 samples collected from these
idiopathic uveitis or endophthalmitis patients [7]. However, this PCR examination could not detect other types of fungal infections. Therefore, as the next step, we have developed a novel PCR examination for broad fungi diagnosis. We attempted to detect whole-genomic fungal DNA in humans by PCR amplification of 28 S rDNA [8].

The new assay targets a part of the 28 S rDNA found in Candida, Aspergillus, Cryptococcus, Mucor, Penicillium, Pichia, Micosporum, Trichophyton, and Scopulariopsis [8]. It is assumed that infectious endophthalmitis related to fungal infection may be caused by various human pathogenic fungi, and the ocular infection may indicate various types of endophthalmitis, such as endogenous, post-

### Table 1 Detection of fungal 28 S rDNA in endophthalmitis and uveitis patients

<table>
<thead>
<tr>
<th>Case no.</th>
<th>Age/gender</th>
<th>Diagnosis</th>
<th>Sample DNA (ng/ml)</th>
<th>Real-time PCR (copies/ml)</th>
<th>Cultures with ocular fluids</th>
<th>Fungal blood test a</th>
</tr>
</thead>
<tbody>
<tr>
<td>24</td>
<td>76/M</td>
<td>Endogenous endophthalmitis</td>
<td>AH 26.1</td>
<td>&lt;10^2</td>
<td>Negative</td>
<td>BDG - 30.2</td>
</tr>
<tr>
<td>30</td>
<td>67/F</td>
<td>Late postoperative endophthalmitis</td>
<td>AH 21.4</td>
<td>2.0 \times 10^4</td>
<td>Candida albicans</td>
<td>BDG - negative</td>
</tr>
<tr>
<td>77</td>
<td>85/M</td>
<td>Endogenous endophthalmitis</td>
<td>VF 41.3</td>
<td>7.6 \times 10^3</td>
<td>Candida spp.</td>
<td>nt</td>
</tr>
<tr>
<td>161</td>
<td>75/M</td>
<td>Endogenous endophthalmitis</td>
<td>VF 57.8</td>
<td>2.8 \times 10^5</td>
<td>Candida albicans</td>
<td>nt</td>
</tr>
<tr>
<td>179</td>
<td>74/F</td>
<td>Endogenous endophthalmitis</td>
<td>AH 29.1</td>
<td>2.6 \times 10^3</td>
<td>Negative</td>
<td>Aspergillus antigen - 36.8</td>
</tr>
<tr>
<td>231</td>
<td>64/M</td>
<td>Endogenous endophthalmitis</td>
<td>VF 105</td>
<td>1.7 \times 10^3</td>
<td>Negative</td>
<td>BDG - 11.9</td>
</tr>
<tr>
<td>268</td>
<td>40/M</td>
<td>Endogenous endophthalmitis</td>
<td>VF 30.2</td>
<td>2.2 \times 10^5</td>
<td>Cryptococcus neoformans</td>
<td>BDG - negative</td>
</tr>
<tr>
<td>326</td>
<td>86/F</td>
<td>Endogenous endophthalmitis</td>
<td>VF 85.7</td>
<td>1.5 \times 10^5</td>
<td>Negative</td>
<td>BDG - 51.7</td>
</tr>
<tr>
<td>355</td>
<td>81/M</td>
<td>Late postoperative endophthalmitis</td>
<td>AH 62.7</td>
<td>7.9 \times 10^6</td>
<td>nt</td>
<td>nt</td>
</tr>
<tr>
<td>359</td>
<td>69/M</td>
<td>Late postoperative endophthalmitis</td>
<td>VF 20</td>
<td>5.1 \times 10^4</td>
<td>Candida spp.</td>
<td>BDG - 36.8</td>
</tr>
<tr>
<td>455</td>
<td>67/M</td>
<td>Primary intraocular lymphoma</td>
<td>VF 41.4</td>
<td>5.0 \times 10^4</td>
<td>nt</td>
<td>nt</td>
</tr>
<tr>
<td>461</td>
<td>63/M</td>
<td>Endogenous endophthalmitis</td>
<td>AH 35.7</td>
<td>&lt;10^2</td>
<td>Negative</td>
<td>BDG - 24.6; C. acbicans</td>
</tr>
<tr>
<td>490</td>
<td>49/M</td>
<td>Endogenous endophthalmitis</td>
<td>AH 43</td>
<td>6.6 \times 10^4</td>
<td>Candida albicans</td>
<td>BDG - 449; C. acbicans</td>
</tr>
</tbody>
</table>

Using broad-range quantitative PCR, fungal 28 S rRNA gene (rDNA) could be detected in 11 ocular samples of ten fungal endophthalmitis cases and one non-infectious case.

AH aqueous humor, nt not tested, PPV pars plana vitrectomy, SA systemic antimycotic (oral or intravenous), TA topical antimycotic, VF vitreous fluids

Fungal blood test levels of β-D-glucan (BDG: pg/ml), detection of fungal antigens (pg/ml), and conventional fungal cultures

### Table 2 Summary of risk factors and therapies in fungal endophthalmitis patients

<table>
<thead>
<tr>
<th>Case no.</th>
<th>Diagnosis</th>
<th>Risk factors</th>
<th>A B</th>
<th>C</th>
<th>Outcome</th>
</tr>
</thead>
<tbody>
<tr>
<td>24</td>
<td>Endogenous endophthalmitis</td>
<td>IVH, peritoneal catheter (+)</td>
<td>SA</td>
<td>PPV, SA</td>
<td>Resolved</td>
</tr>
<tr>
<td>30</td>
<td>Late postoperative endophthalmitis</td>
<td>Vitrectomy, IOL second implant (-)</td>
<td>None</td>
<td>PPV, SA</td>
<td>Resolved</td>
</tr>
<tr>
<td>77</td>
<td>Endogenous endophthalmitis</td>
<td>Diabetes (-)</td>
<td>PPV</td>
<td>SA</td>
<td>Resolved</td>
</tr>
<tr>
<td>161</td>
<td>Endogenous endophthalmitis</td>
<td>Diabetes (-)</td>
<td>PPV</td>
<td>Unknown</td>
<td>Unknown</td>
</tr>
<tr>
<td>179</td>
<td>Endogenous endophthalmitis</td>
<td>Diabetes, pancreas carcinoma, IVH (+)</td>
<td>SA, TA</td>
<td>SA, TA</td>
<td>Resolved</td>
</tr>
<tr>
<td>231</td>
<td>Endogenous endophthalmitis</td>
<td>Gallbladder carcinoma, IVH (+)</td>
<td>PPV, SA</td>
<td>SA</td>
<td>Resolved</td>
</tr>
<tr>
<td>268</td>
<td>Endogenous endophthalmitis</td>
<td>Triamcinolone subtenon injection (-)</td>
<td>PPV</td>
<td>SA</td>
<td>Resolved</td>
</tr>
<tr>
<td>326</td>
<td>Endogenous endophthalmitis</td>
<td>Aortocoronary bypass, IVH (+)</td>
<td>PPV, SA</td>
<td>SA, TA</td>
<td>Resolved</td>
</tr>
<tr>
<td>355</td>
<td>Late postoperative endophthalmitis</td>
<td>PEA + IOL (-)</td>
<td>None</td>
<td>TA</td>
<td>Resolved</td>
</tr>
<tr>
<td>359</td>
<td>Late postoperative endophthalmitis</td>
<td>PEA + IOL (+)</td>
<td>PPV, SA</td>
<td>SA</td>
<td>Resolved</td>
</tr>
<tr>
<td>455</td>
<td>Primary intraocular lymphoma</td>
<td>Lymphoma, steroid use (-)</td>
<td>Methylprednisolone</td>
<td>Methotrexate</td>
<td>Resolved</td>
</tr>
<tr>
<td>461</td>
<td>Endogenous endophthalmitis</td>
<td>Aortocoronary bypass, IVH (+)</td>
<td>SA</td>
<td>SA</td>
<td>Resolved</td>
</tr>
<tr>
<td>490</td>
<td>Endogenous endophthalmitis</td>
<td>Subarachnoid hemorrhage, IVH (+)</td>
<td>PPV, SA</td>
<td>SA, TA</td>
<td>Resolved</td>
</tr>
</tbody>
</table>

A = presence or absence of antimycotic therapy before PCR examination
B = therapy prior to PCR examination
C = therapy after PCR examination

IVH intravenous hyperalimentation, IOL intraocular lens, PEA phacoemulsification and aspiration, PPV pars plana vitrectomy, SA systemic antimycotic (oral or intravenous)
traumatic, post-operative, and ocular surface infection (e.g., corneal ulcer). Furthermore, real-time PCR assays play an important role among molecular genetic screening methods because of the rapid diagnostic outcome. As shown in the current study, a broad-range real-time PCR assay targeting clinically relevant fungal species in one assay is now available.

In two false-negative cases that were clinically suspected of having fungal endophthalmitis (perhaps Candida-associated), our fungal 28 S PCR did not detect any fungal genome in the ocular samples. However, it should be noted that these samples were aqueous humor and not vitreous fluid. If a vitreous sample or a retinal tissue sample had been obtained by biopsy, we might have detected fungal DNA by this PCR method, because endophthalmitis, especially Candida spp., often results from hematogenous dissemination. In cases of postoperative endophthalmitis related to fungal infection, the result from an aqueous humor sample as well as a vitreous sample may be reliable. In fact, two aqueous humor samples were used to detect late postoperative endophthalmitis in the current study. Thus, the type of sample that is collected may be very important for an accurate diagnosis. Depending on the exact clinical setting, a vitreous sample likely offers an optimal diagnosis, since the cultures are usually more accurate. An aqueous sample is, obviously, more easily accessible, but the diagnostic power should be quoted only for the type of sample so as not to confuse expectations and dependency on the results. In cases of fungal endophthalmitis in immunocompetent patients, specific additional antymycotic therapy has been shown to be effective in controlling the ocular inflammation [10–12]. In fact, our PCR-negative immunocompetent patients were finally well controlled by the antymycotic treatment.

In conclusion, we developed a novel protocol for the rapid detection of fungal DNA in ocular samples that was based on fungal species that commonly cause eye disorders. This broad-range real-time PCR method can be utilized for rapid diagnosis of patients who have unknown infectious intraocular disorders. For clinicians to be able to identify the type of fungi, we may need to consider the use of sequence analysis. In the near future, we may be able to determine the fungal species via sequence analysis and rapidly diagnose fungal endophthalmitis; then, we will be able to promptly begin appropriate treatment with antymycotic drugs.

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Competing interests None.

Contributors MO was the principal investigator, designed and performed experiments, and wrote the manuscript. SS designed and conceptualized the study and drafted and edited the manuscript. KW and NS performed PCR assays. MM designed and conceptualized the study and edited the manuscript.

Data sharing statement No additional data.

Ethics approval Ethics approval was provided by the Institutional Ethics Committee of Tokyo Medical and Dental University.

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