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Efficacy of Chromogenic Candida Agar for isolation and presumptive identification of pathogenic yeast species

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ABSTRACT

Chromogenic Candida Agar is a novel differential culture medium that is claimed to facilitate isolation and identification of *Candida albicans*, *Candida tropicalis* and *Candida krusei*. The performance of this medium was evaluated for presumptive identification of 521 yeast strains, representing 23 different species, for detection of specimens containing yeast mixtures, and for direct isolation of yeast from blood cultures. All yeasts grew well on the medium following a 48-h incubation period at 37°C, and distinctive colonies were produced by *C. albicans*, *C. tropicalis*, *C. krusei*, *Candida guilliermondii*, *Saccharomyces cerevisiae*, *Trichosporon mucoides* and *Geotrichum capitatum*. The sensitivity and specificity of the medium exceeded 99.4% for each of these species. The medium provided some indication of the presence of *Candida dubliniensis* and *Candida pulcherrima*, and allowed the identification of polyfungal samples in 89.4% of the yeast mixtures. Finally, direct isolation on the medium from blood cultures that were positive for yeast according to Gram's stain (n = 42) showed that the expected colour and morphology of each species were not altered in the presence of blood.

Keywords *Candida*, Chromogenic Candida Agar, diagnosis, identification, selective medium, yeasts

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INTRODUCTION

Yeast infections require prompt diagnosis to allow the early initiation of appropriate antifungal therapies. The need for rapid identification of the pathogen and the difficulty in detecting mixed cultures on the traditional Sabouraud glucose agar have led to the design of commercial isolation media intended to differentiate yeast species on the basis of colony colour [1–5]. Currently, three chromogenic agars are widely used in clinical mycology laboratories for the detection and presumptive identification of *Candida* spp., particularly *Candida albicans*, i.e., Candiselect 4 (Bio-Rad, Marnes La Coquette, France), Candida ID agar (bioMérieux, Marcy l'Etoile, France) and CHROMagar Candida (CHROMagar Company, Paris, France). Candiselect 4 allows presumptive identification of the related species *C. albicans/Candida dubliniensis* (pink–purple colonies on this medium) and their discrimination from *Candida tropicalis*, *Candida krusei* and *Candida glabrata*, which form turquoise colonies [5].

Candida ID agar is based on a chromogenic indolyl glucosaminide substrate, which is hydrolysed by *C. albicans* and *C. dubliniensis* to yield a blue product [2,6,7]. *Trichosporon* spp. also form blue colonies on this medium, but can be differentiated from *C. albicans* and *C. dubliniensis* by their macroscopic morphology [2]. A new version of this medium (Candida ID agar 2; bioMérieux) has been suggested to differentiate *C. albicans* from *C. dubliniensis* [8]. CHROMagar Candida contains a chromogenic β-glucosaminidase substrate that reacts with species-specific enzymes to give colonies with different colours. This medium allows identification of *C. albicans*, *C. tropicalis*, *C. krusei*, and *Trichosporon* spp. [4]. Some reports have suggested that CHROMagar Candida can provide an indication of the presence of *C. glabrata* [9,10] and discrimination of *C. dubliniensis* from *C. albicans* [11], but differentiation of these species on

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this medium remains somewhat controversial [4,9,12,13]. CHROMagar Candida enables good differentiation of yeast species in mixed cultures [14], in direct isolation of *Candida* spp. from blood cultures [15], and in the prediction of susceptibility to fluconazole for many *Candida* strains isolated from blood cultures [16].

The new commercial Chromogenic Candida Agar (CCA; Oxoid, Basingstoke, UK) incorporates 5-bromo-4-chloro-3-indolyl/*N*-acetyl-β-D-glucosaminide and 5-bromo-6-chloro-3-indolyl phosphate *p*-toluidine salt as chromogenic substrates to detect yeast hexosaminidase and alkaline phosphatase activity, respectively. A previous report describing the use of this medium has shown that CCA is highly selective for yeasts and allows presumptive identification of *C. albicans*, *C. tropicalis* and *C. krusei*, and that it can be useful in indicating the polyfungal content of clinical samples [17]. The purpose of the present study was to evaluate the performance of CCA for the isolation and presumptive identification of a large number of yeast species, including less common agents of mycosis and the newly described species *Candida orthopsilosis* and *Candida metapsilosis*. In addition, the study investigated whether CCA could facilitate the detection of different colony types when seeded with various combinations of yeast suspensions, and the usefulness of CCA for direct isolation of yeasts from blood cultures.

MATERIALS AND METHODS

Strains

In total, 506 yeast isolates were recovered from specimens submitted to the laboratory from different clinical units within Pisa University Hospital (Pisa, Italy) and identified according to the API ID32C yeast identification panel (ATB Fungus System; bioMérieux) or the ID-YST card system (Vitek System; bioMérieux). *C. orthopsilosis* and *C. metapsilosis* were identified by screening all isolates identified phenotypically as *Candida parapsilosis* with a DNA-based molecular test [18]. In brief, a 716-bp fragment of the secondary alcohol dehydrogenase gene (*SADH*) was amplified, purified and digested with *Ban*I. Differential digestion profiles were then used to identify the three species, since *C. parapsilosis*, *C. metapsilosis* and *C. orthopsilosis SADH* amplicons contain one, three and no *Ban*I restriction sites, respectively. Reference strains included *C. krusei* ATCC 6258, *Candida guilliermondii* ATCC 6260, *C. albicans* ATCC 10231, *Candida rugosa* DSM 70761, *C. glabrata* ATCC 90030, *C. tropicalis* ATCC 4563, *Candida lusitaniae* DSM 70102, *Candida pulcherrima* ATCC 22032, *Candida famata* DSM 70590, *C. parapsilosis* ATCC 22019, *C. orthopsilosis* ATCC 56139, *C. metapsilosis* ATCC 56143, *Saccharomyces cerevisiae* ATCC 9763, *Trichosporon mucoides* ATCC 204094 and *Cryptococcus neoformans* ATCC 90112.

Culture conditions

Yeast strains were cultured routinely on Sabouraud dextrose agar (SDA; Becton Dickinson, Franklin, NJ, USA) and were stored in Sabouraud broth (Becton Dickinson) containing glycerol 30% v/v at −80°C. CCA was obtained from Oxoid. Before being tested on this medium, frozen yeast strains were thawed and subcultured on SDA. Yeast suspensions were then streaked to form single colonies on CCA. In a pilot study, 136 cultures inoculated on CCA were incubated at both 30°C and 37°C and were examined after 24, 48 and 72 h, in order to establish the optimum growth conditions. This preliminary evaluation revealed that the optimal temperature for yeast growth and colour development was 37°C. Although all species, with the exception of *S. cerevisiae*, grew well after 24 h at 37°C, only some species developed a distinctive colony colour at that time. The definitive colony appearance was obtained for all species only after incubation for 48 h, and no difference in colony appearance was detected when the incubation was extended to 72 h. Therefore, analysis of colonies on CCA was carried out after incubation for 24 and 48 h at 37°C. Different lots of CCA ($n = 4$) were used in these experiments, but no variations in colony colour and yeast growth were observed. All plates were examined visually by three independent investigators for colony colour, size, texture and the presence of colour diffusion into the surrounding agar. Colony colours were described according to the Pantone Colour Formula Guide. Sensitivity was expressed as the number of true-positives × 100/number of true-positives plus the number of false-negatives, and specificity was expressed as the number of true-negatives × 100/number of true-negatives plus the number of false-positives.

For the analysis of mixed cultures, individual strains were resuspended in NaCl 0.85% w/v to a McFarland standard of 2.0, and mixtures of two or three yeast species were prepared by mixing equal volumes of each yeast suspension. Aliquots (1 μL) of polyfungal suspensions were streaked for isolation on duplicate sets of CCA plates. Plates were incubated at 37°C and the number of distinguishable colony types was recorded after incubation for 48 h.

Direct isolation from blood cultures

The blood cultures used in this study were submitted to the laboratory from different units of the Pisa University Hospital. Blood samples were collected and inoculated in BACTEC PLUS Aerobic/F and Anaerobic/F bottles and were monitored using the BACTEC 9240 system (Becton Dickinson). Positive cultures identified by the system that contained yeast according to Gram's stain were then inoculated on CCA by withdrawing a 100-μL aliquot from each positive bottle and streaking with a loop. Plates were incubated at 37°C and inspected visually after 24 and 48 h. In parallel, a control aliquot of the blood culture was plated on SDA and the resulting yeast colonies were identified using the API ID32C yeast identification panel or the ID-YST card system.

RESULTS AND DISCUSSION

Appearance of colonies on CCA

The medium supported the growth of all the clinical isolates and reference strains. A wide

Table 1. Appearance of yeast colonies after growth on Chromogenic Candida Agar for 24 and 48 h at 37°C

Species	No. of isolates	Isolates identified after incubation for 24/48 h	Colour	Colour number[a]
Candida albicans	159	140/159	Green	377, 3405, 359
Candida dubliniensis	4	4/4	Bluish green	3282, 329
Candida krusei	40	35/40	Pink[b]	481, 4735, 435
Candida tropicalis	40	38/40	Dark blue	302, 308
Candida guilliermondii	24	17/24	Blackberry wine	4715, 4735, 4745, 7525
Candida glabrata	72	72/72	Light to dark brown	7403, 465
Candida parapsilosis	24	24/24	Light brown	7403, 1205, 1215
Candida orthopsilosis	11	11/11	Light brown	7403, 1205, 1215
Candida metapsilosis	7	7/7	Light brown	7403, 1205, 1215
Candida lusitaniae	19	19/19	Light to dark brown	7403
Candida inconspicua	2	2/2	Light brown	1205, 1345, 134
Candida norvegensis	16	16/16	Light brown	1205, 1345, 134
Candida famata	23	23/23	Light to dark brown	466, 4625
Candida kefyr	5	5/5	Light brown	135, 1355
Candida lipolytica	4	4/4	Light brown	7403, 1205
Candida pelliculosa	3	2/2	Light to dark brown	7504, 407, 408
Candida rugosa	1	0/1	Light brown	7403, 1205, 1215
Candida pulcherrima	4	4/4	Light brown, pink halo	470, 415
Geotrichum capitatum	8	8/8	Dark pink[c]	4755
Saccharomyces cerevisiae	28	9/27	Violet[d]	519, 5145
Rhodotorula mucilaginosa	12	11/12	Salmon-pink	170, 178
Trichosporon mucoides	13	13/13	Bluish green → brown	312, 322, 126[e]
Cryptococcus neoformans	1	1/1	Light brown	7403
Cryptococcus albidus	1	0/1	Light brown	7403

[a]Colour numbers from the Pantone Colour Formula Guide.
[b]Wide, rough, dry colonies with a dark centre.
[c]Small, rough colonies different from those of *C. krusei*.
[d]One isolate developed brown colonies.
[e]Colonies at the end of the streak have a brownish appearance.

variety of colony colours was seen, some of which were species-specific (Table 1). The white/opaque background of CCA seemed to allow good discrimination among colonies with relatively similar hues.

All the *C. albicans* isolates ($n = 159$) formed green colonies after incubation for 48 h on CCA (Fig. 1a). This hue was distinctive for this species and, among the other species tested, only *C. dubliniensis* gave a similar colour (Fig. 1a). Isolates of *C. dubliniensis* ($n = 4$) developed dark bluish green colonies, and two isolates could not be distinguished from *C. albicans* by the independent investigators. Therefore, as shown previously for CHROMagar Candida [13], a dark bluish green appearance on CCA may be taken as an indication of the presence of *C. dubliniensis*, but should not be used as the sole criterion for identification. The sensitivity of CCA for the identification of *C. albicans* was 88% and 100% after incubation for 24 and 48 h, respectively, with a specificity of 99.4% at both incubation times.

All *C. krusei* ($n = 40$), *C. tropicalis* ($n = 40$) and *C. guilliermondii* ($n = 24$) isolates formed typical colonies that were easily differentiated from those of other yeasts (Table 1). *C. krusei* colonies were

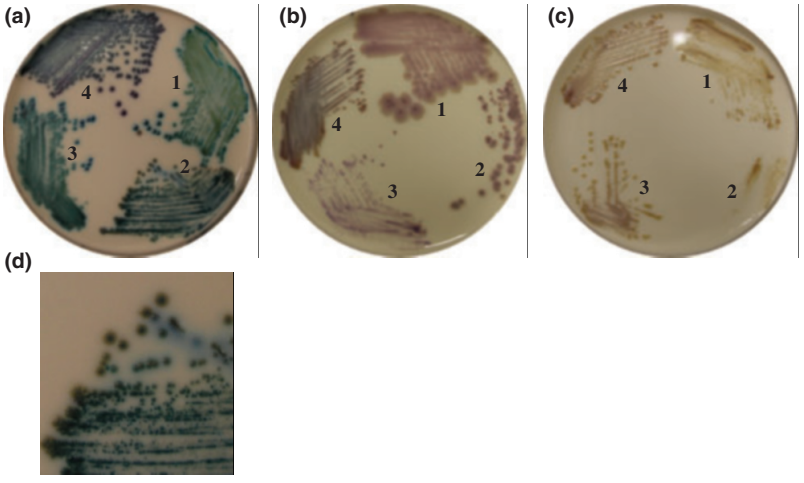

Fig. 1. Appearance of yeast colonies on Chromogenic Candida Agar. (a) 1, *Candida albicans*; 2, *Trichosporon mucoides*; 3, *Candida dubliniensis*; 4, *Candida tropicalis*. (b) 1, *Candida krusei*; 2, *Geotrichum capitatum*; 3, *Saccharomyces cerevisiae*; 4, *Candida guilliermondii*. (c) 1, *Candida parapsilosis*; 2, *Cryptococcus albidus*; 3, *Candida glabrata*; 4, *Candida lusitaniae*. (d) Enlarged *T. mucoides* colonies.

dry, rough, pink and spreading with a dark centre (Fig. 1b). *C. tropicalis* formed dark blue colonies (Fig. 1a) and *C. guilliermondii* formed blackberry wine-coloured colonies (Fig. 1b) that were not confused with those of *C. krusei*, *Geotrichum capitatum* or *S. cerevisiae* (the only three species that gave colonies with a similar hue). One isolate of *Candida pelliculosa* was not distinguished from *C. guilliermondii*. The specificity and sensitivity of CCA for the identification of *C. krusei*, *C. tropicalis* and *C. guilliermondii* were very high, with a specificity of 100% for *C. krusei* and *C. tropicalis*, and 99.8% for *C. guilliermondii*, after incubation for 48 h. The sensitivity of CCA at 24 h was 87.5%, 95.0% and 70.8% for *C. krusei*, *C. tropicalis* and *C. guilliermondii*, respectively, and reached 100% after 48 h for all species.

These findings indicate that CCA allows presumptive identification of *C. albicans*, *C. tropicalis* and *C. krusei* with high sensitivity and specificity, particularly after incubation at 37°C for 48 h. Although a greater number of yeast species were considered in the present study, the results essentially confirmed data reported previously concerning the usefulness of CCA for the identification of these three species [17]. Based on the available data, it therefore seems that CCA can be used to reliably differentiate *C. albicans*, *C. tropicalis* and *C. krusei*, with a discriminatory power comparable to that of CHROMagar Candida [4,9]. However, the present study also revealed that CCA permits the identification of *C. guilliermondii*. Notably, CCA appears to be the only commercial medium that allows the identification of this species, which ranks sixth among the most frequently isolated *Candida* spp. and shows reduced susceptibility to fluconazole [19].

CCA did not enable the differentiation of *C. glabrata* (n = 72), *C. parapsilosis* (n = 24), *C. orthopsilosis* (n = 11), *C. metapsilosis* (n = 7), *C. lusitaniae* (n = 19), *Candida inconspicua* (n = 2), *Candida norvegensis* (n = 16) and *C. famata* (n = 23), or the relatively uncommon *Candida kefyr* (n = 5), *Candida lipolytica* (n = 4), *C. pelliculosa* (n = 3) and *C. rugosa* (n = 1), all of which formed light to dark brown colonies (Fig. 1c; Table 1). Smaller, albeit indistinguishable, colonies were also produced by *Cryptococcus neoformans* and *Cryptococcus albidus* (Fig. 1c). *C. pulcherrima* (n = 4) gave light brown colonies, but with a distinctive pink halo (Table 1). Therefore, the formation of a pink halo around brown colonies on CCA could be considered to be an indication of the presence of *C. pulcherrima*.

The lack of discrimination observed for many *Candida* spp. partially limits the usefulness of CCA in the clinical mycology laboratory. Indeed, *C. glabrata* and *C. parapsilosis*, two of the species involved most frequently in candidaemia [20], could not be differentiated using this medium. In addition, no discrimination was observed for less common *Candida* spp., e.g., *C. famata*, *C. lusitaniae*, *C. lipolytica*, *C. pelliculosa* and *C. kefyr*, which have been reported to be responsible for *c.* 5% of *Candida* bloodstream infections [20].

T. mucoides isolates (n = 13) formed bluish green–brown colonies that were easily distinguishable from those of *C. albicans* and *C. dubliniensis* (Fig. 1a). Indeed, while *T. mucoides* colonies were green in the quadrant showing most growth, isolated colonies developed a distinctive brown hue (Fig. 1d). Since the hexosaminidase activity is responsible for 5-bromo-4-chloro-3-indolyl/*N*-acetyl-β-D-glucosaminide conversion to a green–blue product on CCA, the green *T. mucoides* colonies in the area of confluent growth could be explained in terms of an accumulation of this enzyme, which may only be produced at a low concentration by isolated colonies.

The colour and morphology of *S. cerevisiae* colonies on CCA only became typical after incubation at 37°C for 48 h; most *S. cerevisiae* strains formed very small, pinpoint colonies with a distinctive violet colour (Fig. 1b), but one *S. cerevisiae* isolate formed brown colonies that were indistinguishable from those produced by several other yeast species. The specificity and sensitivity of CCA for the identification of *S. cerevisiae* were 100% and 96.4%, respectively, after incubation for 48 h.

G. capitatum (n = 8) isolates produced dark pink, fimbriate and rough colonies that were easily distinguishable in colour and morphology from those of *C. krusei* (Fig. 1b), while salmon-pink, mucoid colonies were produced by all *Rhodotorula mucilaginosa* isolates (n = 12).

The finding that CCA, in addition to *Candida* spp., can allow identification of yeasts such as *T. mucoides*, *G. capitatum* and *S. cerevisiae* was considered to be of diagnostic relevance. These species are reported increasingly as agents of invasive infections, especially in immunocompromised or critically-ill patients. Disseminated infections caused by *Trichosporon* spp. and

G. capitatum [21] are emerging and cause frequently fatal mycoses among neutropenic patients, particularly those who have received drugs with cytotoxic and immunosuppressive effects for haematological malignances [21]. Although considered previously to be an occasional digestive commensal, *S. cerevisiae* has now been shown to behave as an opportunistic pathogen that is responsible for sporadic systemic infections [22].

CCA for discrimination of yeasts from polyfungal suspensions

In total, 47 suspensions were prepared, 44 of which contained two species and three of which contained three species (Table 2). CCA allowed the identification and differentiation of yeast mixtures in 42 (89.36%) samples. Most of these samples contained yeast species that were easily recognised because of their different colony colours and textures (Table 2). Fig. 2a shows the appearance of colonies produced by a mixture containing *C. dubliniensis*, *C. orthopsilosis* and *R. mucilaginosa* after incubation at 37°C for 48 h on CCA. Remarkably, *C. albicans* and *C. dubliniensis* were easily distinguished in two different mixtures, by all investigators, as shown in Fig. 2b. The five mixtures that were not detected on CCA contained *C. glabrata/C. kefyr* ($n = 2$), *C. lusitaniae/C. lipolytica* ($n = 2$) and *C. kefyr/C. lusitaniae/C. parapsilosis* ($n = 1$). The similar light brown appearance of the colonies produced by all of these species did not allow the polyfungal nature of the culture to be recognised. However, in the case of the mixture containing three species, the independent investigators recorded the presence of two distinguishable colony types.

Table 2. Detection of multiple yeast species from polyfungal suspensions using Chromogenic Candida Agar

Species	No. of mixed cultures	No. of distinct colony types
Candida albicans/Candida dubliniensis	2	2
C. albicans/Candida krusei	4	2
C. albicans/Candida tropicalis	4	2
C. albicans/Candida guilliermondii	3	2
C. albicans/Candida glabrata	3	2
C. albicans/Candida parapsilosis	2	2
C. albicans/Trichosporon mucoides	3	2
C. dubliniensis/C. tropicalis	2	2
C. krusei/Rhodotorula mucilaginosa	2	2
C. krusei/Geotrichum capitatum	2	2
C. glabrata/Candida kefyr	2	1
Candida lusitaniae/Candida lipolytica	2	1
C. lusitaniae/C. guilliermondii	2	2
C. glabrata/C. tropicalis	2	2
C. glabrata/C. krusei	3	2
C. dubliniensis/T. mucoides	1	2
C. glabrata/C. parapsilosis	3	2
Saccharomyces cerevisiae/T. mucoides	2	2
C. dubliniensis/Candida orthopsilosis/R. mucilaginosa	1	3
C. albicans/C. orthopsilosis/C. tropicalis	1	3
C. kefyr/C. lusitaniae/C. parapsilosis	1	2

Isolation from blood cultures

In total, 42 blood cultures positive for yeast species were collected and analysed on CCA. In parallel, the isolates were cultured on SDA and identified phenotypically. All isolates identified as *C. parapsilosis* by biochemical testing were subsequently analysed to determine whether they belonged to the species *C. metapsilosis* or *C. orthopsilosis*. The distribution of clinical isolates comprised *C. albicans* ($n = 21$), *C. glabrata* (9), *C. parapsilosis* (1), *C. orthopsilosis* (2), *C. tropicalis* (4), *C. krusei* (3), *C. pulcherrima* (1) and *S. cerevisiae* (1). In general, there was a more profuse yeast growth on SDA at 24 h than on CCA, but a comparable number and size of colonies were observed on the two media after incubation for 48 h. All yeasts derived from blood cultures grew well on CCA after incubation for 48 h. Yeasts growing over the initial quadrant of streaking often showed a hue slightly different from that seen in the other

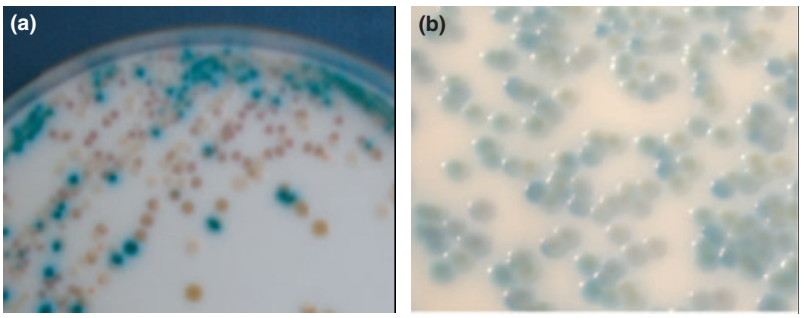

Fig. 2. Growth of polyfungal cultures on Chromogenic Candida Agar. (a) *Candida dubliniensis* (bluish green colonies), *Candida orthopsilosis* (light brown colonies) and *Rhodotorula mucilaginosa* (salmon-pink colonies). (b) *Candida albicans* (green colonies) and *C. dubliniensis* (bluish green colonies).

quadrants. However, no significant difference was observed in the colour or general appearance of colonies produced by each yeast species isolated from blood as compared with those produced by frozen yeast strains. Although the presence of haem pigment could potentially affect the typical colony appearance on CCA, the expected colour used to identify each species was not altered by direct isolation from blood. Therefore, these findings indicate that CCA can also be used for primary isolation and presumptive identification of yeasts from blood cultures.

CONCLUSIONS

CCA was found to allow the growth of most clinically relevant yeasts, and to allow presumptive identification of *C. albicans*, *C. tropicalis*, *C. krusei*, *C. guilliermondii*, *S. cerevisiae*, *T. mucoides* and *G. capitatum*. CCA also facilitated recognition of specimens containing mixtures of yeast species, and was also suitable for primary isolation of yeasts from blood cultures. The medium did not allow discrimination of *C. glabrata* and *C. parapsilosis*, two of the most common agents of candidiasis, as well as several other less common *Candida* spp. Although this lack of discriminatory activity is a disadvantage, CCA appears to be an acceptable source medium for primary isolation and presumptive identification of a number of common yeast species.

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