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ORIGINAL ARTICLE

Fungal diversity and *Aspergillus* in hospital environments

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Abstract

Introduction and objective. Nosocomial invasive fungal infections, particularly aspergillosis, are an increasing problem in immunocompromised patients. The presented study evaluates fungal diversity and the presence of *Aspergillus* in air samples from two hospitals.

Materials and methods. Over the course of one year (rainy and dry seasons), the air was sampled from three areas in two hospitals (1 and 2) using a single-stage Andersen viable particle sampler (Thermo Scientific, Waltham, MA, USA). The fungi were identified by macro- and micromorphology, and the number of colony forming units (CFU)/m^3 air and their richness, abundance, and diversity were determined. Isolates *Aspergillus* genus were characterized by their thermotolerance.

Results. The CFU/m^3 air was similar at both hospitals during the two seasons, but different between the sampled areas. Results showed 10 fungal genera for hospital 1, and 8 for hospital 2. The most abundant were *Penicillium*, *Cladosporium* and *Aspergillus*. The thermotolerance test confirmed the identification of *A. fumigatus* section *Fumigati*. The highest growth rate was found in *Aspergillus* section *Nigri*.

Conclusion. Determining the fungal diversity in the two hospitals was important because all the species have the potential to be pathogenic, especially the section *Fumigati*.

Key words

fungal diversity, *Aspergillus* spp., airborne, thermotolerance

INTRODUCTION

Exposure to bioaerosols is one of the greatest threats to public health because of the adverse effects on humans, animals, and plants [1, 2, 3]. Bioaerosols are aerial suspensions of particles from living organisms, microorganisms or other biological materials [4, 5], the bioaerosols can be dispersed over long distances by air currents and then inhaled, ingested, or otherwise contacted by humans [3]. Fungi are an important component of the bioaerosols of the air and are widely distributed in soil, water, and decaying vegetation [6], and fungi can change benign superficial infections into invasive mycoses [6–11]. Most of the fungi in the environment, both indoors and outdoors, belong to the divisions Ascomycota, Basidiomycota, and mitosporic fungi [12], with *Penicillium*, *Aspergillus*, *Cladosporium*, and *Alternaria* the most abundant genera [13]. In intramural environments such as those of hospitals, the diversity of fungi is similar, but the concentration and abundance of conidia is associated with their growth in building materials, food, pots, bedding, dust, paint, etc.; under suitable temperature and moisture conditions, conidia grow and sporulate on these substrates, thus constituting a significant source of conidia and hyphal fragments in the air. Numerous studies have been conducted on the adverse effects on human health caused by fungi in intramural and extramural environments [11]. In most of these studies, the single-stage Andersen viable particle sampler (Thermo Scientific; Waltham, MA, USA) is the most frequently used air sampler [14–17] in the detection of colony forming units (CFU) per cubic meter of air, after which identification of the fungi is performed by culture or by molecular techniques [14, 15, 17, 18]. Studies have shown that the highest percentage of nosocomial infections is caused by fungi such as *Candida albicans* and the previously mentioned filamentous fungi [19–22]. A genus that has emerged most often as an important cause of morbidity and high mortality in immunocompromised patients is *Aspergillus* [23, 24]. Invasive aspergillosis (IA) and other infections caused by filamentous

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fungi occur in different groups of immunocompromised patients, including patients with haematologic malignancies [25] who have undergone transplantation [26], patients with congenital immunodeficiencies [27], and patients undergoing immunosuppressive drug therapy [28]. Therefore, the objective of this study was to assess fungal diversity and the presence of airborne fungi at two hospitals in Mexico City.

MATERIALS AND METHOD

Sampling area. Sampling was conducted from May – October 2012 (rainy season) and November 2012 – April 2013 (dry season) in two hospitals located in Mexico City: a specialty hospital, which houses nearly 1,400 patients per month, and the Institute of Ophthalmology, which can house 100 patients; the hospitals were designated 1 and 2, respectively. The areas sampled in hospital 1 were haematology, adult intensive care, and paediatric intensive care units, whereas the areas sampled in hospital 2 were the operating theatre, recovery room, and outpatient room. The sampling of air in different areas was always performed on the same day (Monday and Wednesday) and at the same time (10:00).

Air sampling procedure. Air samples were obtained with a single-stage Andersen viable particle sampler (Thermo Scientific; Waltham, MA, USA). This system impacts the microorganisms from the environment onto Sabouraud-agar medium (Bioxón, D.F., Mexico). The sampler was placed at a height of 1.5 m from the floor, in the center of each sampling area, and each sample was collected with a vacuum flow of 28.3 L/min for 15 min. Three samples were collected from each sampling area [29].

Fungal examination of airborne samples. After three days of incubation at 28 °C, all the plates were examined and the colonies counted. For each sampled area, the average number of all the colonies grown on the three Sabouraud-agar plates was calculated, and the number of CFU/m^3 air determined according to the manufacturer's protocol sampler. In addition, the CFU/m^3 air was calculated separately for each different colony observed on the plates. All the different colonies were identified by their macro and micromorphologies. The isolates grown on Sabouraud-agar at 28 °C for 4–7 days were observed to identify the typical morphology of each fungus, including the colour and colonial texture. The micromorphological characteristics of all isolates were analyzed using the microculture method of Riddell [30]. The isolates with characteristics compatible with *Aspergillus* species were inoculated and incubated at 25 °C in Czapek-Dox agar (Becton Dickinson, MD, USA) and potato dextrose agar (Bioxon) to identify the species using taxonomic keys [31]. Furthermore, the isolates identified in the different sections of *Aspergillus* were tested in assays of thermotolerance to corroborate those belonging to *A. fumigatus* section *Fumigati*. The assays were conducted as described by Frías-De-León et al. [32]. A suspension of 5 x 10^3 conidia/mL was prepared with PBST (phosphate buffered saline solution with tween 20) using a Neubauer chamber and inoculated in triplicate for each temperature (28 and 50 °C). Growth rate (GR) of all *Aspergillus* isolates studied was determined according to the model of Baranyi and Roberts [33].

Fungal diversity. The richness and abundance of organisms were determined in each area sampled at the two hospitals. The richness was obtained based on the number of species isolated in each sampling area; the abundance was determined by the number of occurrences of each microorganism in each area divided by the total CFU of each area and then multiplied by 100. The fungal diversity was calculated for each hospital based on a matrix of the presence and abundance of the species using the Shannon-Wiener (*H*) and Simpson indices and the software PAST version 1.89 (http://folk.uio.no/ohammer/past) [34].

Statistical analysis. Analysis of variance (ANOVA) was performed to determine if there were statistically significant differences in aero-conidial concentration (CFU/m^3) between the dry and cold seasons of each sampling area, and between the hospitals. All tests were conducted at a significance level of 5% using the software NCSS ver. 7.0 (www.ncss.com).

RESULTS

Quantification of CFU/m^3 in hospitals 1 and 2. A total of 432 air samples obtained in the two hospitals were studied. At both hospitals, the concentration of airborne fungi was similar, and no significant differences in the levels of fungi (CFU/m^3) between the dry and rainy seasons ($p>0.05$) (Tab. 1) were observed.

Table 1. Colony forming units (CFU) in each area sampled in hospitals 1 and 2

Hospital	Sampling area	Rainy season CFU/m^3 ± SD	Dry season CFU/m^3 ± SD
1	Haematology	85.98 ± 13.87	97.09 ± 8.55
1	Adult intensive care unit	43.51 ± 9.37	40.22 ± 10.03
1	Paediatric intensive care unit	41.24 ± 8.09	43.75 ± 6.99
2	Outpatient room	56.30 ± 14.77	53.79 ± 10.24
2	Recovery room	50.57 ± 13.99	53.59 ± 6.66
2	Operating theatre	8.53 ± 2.06	8.95 ± 2.69

SD = Standard Deviation

At hospital 1, the highest concentration of conidia in the air (CFU/m^3) was recorded as follows (in descending order): haematology unit, adult intensive care ward, and paediatric intensive care ward. The differences in CFU/m^3 between the three areas sampled in hospital 1 were statistically significant ($p<0.05$). At hospital 2, the highest concentration of CFU/m^3 in the air was recorded as follows (in descending order): outpatient room, recovery room, and operating theatre. The differences in CFU/m^3 between the three areas sampled in hospital 2 were also statistically significant ($p < 0.05$) (Tab. 1).

Identification of fungi isolated in hospitals 1 and 2 by macromorphology and micromorphology. At hospital 1, the fungi detected were: *Acremonium* sp., *Alternaria* sp., *Aspergillus* sp., *Aspergillus* section *Flavi*, *Aspergillus* section *Fumigati*, *Aspergillus* section *Nigri*, *Aspergillus* section *Terrei*, *Cladophialophora bantiana*, *Cladosporium* sp., *Fusarium proliferatum*, *Mucor* sp., *Paecilomyces* sp., *Penicillium* sp., *Rhizopus* sp., *R. stolonifer* and *Trichoderma* sp. (Fig. 1). At hospital 2, the fungi detected were: *Alternaria* sp., *Aspergillus*

sp., *A.* section *Flavi*, *A.* section *Fumigati*, *A.* section *Nigri*, *A.* section *Terrei*, *Cladosporium* sp., *Mucor* sp., *Paecilomyces* sp., *Penicillium* sp., *P. griseofulvum*, *Trichoderma* sp., *Rhizopus* sp., and *R. stolonifer* (Fig. 2).

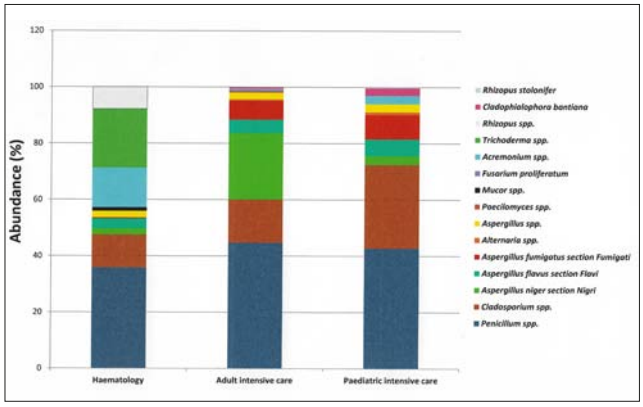

Figure 1. Richness and abundance of fungi in the sampled areas of hospital 1

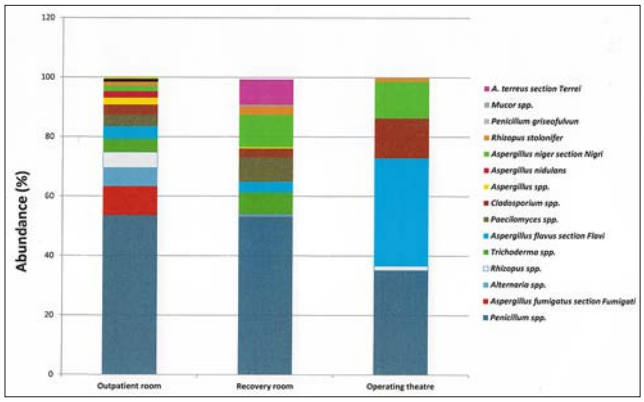

Figure 2. Richness and abundance of fungi in the sampled areas of hospital 2

Fungal diversity. The fungal diversity in hospital 1 showed a richness of fungi distributed as follows: 7 genera and 6 species were found in the adult intensive care unit, 6 genera and 5 species in the paediatric intensive care unit, and 7 genera and 5 species in the haematology unit. In hospital 2: 8 genera and 6 species in the outpatient consultation room, 8 genera and 5 species in the recovery room, and 4 genera and 3 species in the operating theatre (Figs. 1 and 2).

The most abundant fungi sampled in each area of hospital 1 were *A.* section *Flavi*, *A.* section *Fumigati*, *A.* section *Nigri*, *Cladosporium* sp., and *Penicillium* sp. in the pediatric intensive care unit; *Acremonium* sp., *Cladosporium* sp., *Penicillium* sp., *Rhizopus* sp. and *Trichoderma* sp. in haematology; *A.* section *Flavi, A.* section *Fumigati, A.* section *Nigri*, *Cladosporium* sp., and *Penicillium* sp. in adult intensive care (Fig. 1). The most abundant fungi sampled in each area of hospital 2 were: *Alternaria* spp., *A.* section *Flavi*, *A.* section *Fumigati*, *Penicillium* sp., *Rhizopus* sp., and *Trichoderma* sp. in outpatient consultation; *A.* section *Nigri*, *A.* section *Terrei*, *Paecilomyces* sp., *Penicillium* sp. and *Trichoderma* sp. in the recovery room; *A.* section *Flavi*, *A.* section *Nigri, Cladosporium* sp. and *Penicillium* sp. in the operating theatre (Fig. 2). The diversity values estimated by the Shannon-Wiener and Simpson indices revealed a slightly higher diversity in hospital 1 (Tab. 2).

Table 2. Fungal diversity in the different sampled areas in hospitals 1 and 2

Hospital 1			Hospital 2		
Sampled area	Simpson Index	Shannon Index	Sampled area	Simpson Index	Shannon Index
AICU	0.7784	1.822	OPR	0.6593	1.668
HAEM	0.7822	1.942	RR	0.6494	1.677
PICU	0.7915	1.879	OT	0.6172	1.204

AICU = Adult intensive care unit; HAEM = Haematology; PICU = Paediatric intensive care unit; OPR = Outpatient room; RR = Recovery room; OT = Operating theatre.

All the isolates of the genus *Aspergillus* from hospital 1 grew at 28 °C (Tab. 3). The GR for the *A.* section *Fumigati* isolates was in the range of 13.90–19 mm/day and for *A.* section *Nigri* was in the range of 18.13–25.44 mm/day. The GR for *A.* section *Flavi* was in the range of 15.96–19.08 mm/day and for *A.* section *Terrei* was in the range of 8.17–8.87 mm/day. All the *Aspergillus* isolates from hospital 2 grew at 28 °C. The GR for *A.* section *Fumigati* isolates was in the range of 14.79–22.39 mm/day and for *A.* section *Nigri* was in the range of 20.49–25.69 mm/day. The GR for *A.* section *Flavi* was in the range of 14.88–22.52 mm/day and for *A.* section *Terrei* was in the range of 9.20–9.57 mm/day.

Only the isolates from hospitals 1 and 2 identified as *A.* section *Fumigati* grew at 50 °C (Tab. 3). Isolates from hospital 1 presented a GR of 0.88–21.93 mm/day, whereas isolates from hospital 2 presented a range of 2.61–29.21 mm/day (Tab. 3), showing statistically significant differences ($p<0.05$) among their GR, both among and between isolates of hospital 1 and hospital 2.

Table 3. Growth rate of *Aspergillus* spp. isolates

Hospital	Section	Growth rate (mm/day) ± SD	
		28°C	50°C
1	*Fumigati*	16.95 ± 1.63	6.53 ± 5.13
1	*Nigri*	22.95 ± 1.92	NA
1	*Flavi*	17.07 ± 1.19	NA
1	*Terrei*	8.54 ± 0.35	NA
2	*Fumigati*	17.94 ± 2.16	12.61 ± 7.13
2	*Nigri*	23.04 ± 1.39	NA
2	*Flavi*	18.34 ± 2.72	NA
2	*Terrei*	9.38 ± 0.26	NA

SD = Standard deviation; NA = Not applicable

DISCUSSION

In recent decades, there has been a global increase in nosocomial fungal infections because of advances in increasingly effective, but also more aggressive, medical and surgical therapies [35]. Invasive fungal diseases caused by filamentous fungi are associated with high morbidity and mortality, which is partially because of the difficulty in making an early diagnosis, thus resulting in a delay in starting appropriate treatment. Although fungi have several routes of entry into the host, the most common is through inhalation of propagules; thus, maintaining good air quality in critical areas of hospitals is required to reduce the incidence of invasive fungal infections. Therefore, the presented study is aimed at detecting airborne fungi in the environment of

different areas of two hospitals (1 and 2) to illustrate the occurrence of fungal species. The results obtained are in accordance with the results of other aeromycology studies from Mexico and Brazil [36–38].

During the sampling period, the concentration of fungi in the air (CFU/m^3) at both hospitals was similar for both the dry and rainy seasons, regardless of the conditions or structure of each sampling area. The CFU/m^3 in the sampled areas of the two hospitals showed averages above 40.22 CFU/m^3, with the exception of the operating theatre in hospital 2, which produced values of 8.53 and 8.95 for the rainy and dry seasons, respectively. The fungal burden found in the sampled areas of the two hospitals is inconsistent with that reported in other countries [28, 29]; however, the CFU/m^3 in the operating theatre is consistent with such reports (5–10 CFU/m^3), which can be explained by the stringent hygienic measures in operating theatres. Similarly, there are no established criteria at the institutional level in Mexico regarding the ranges of CFU/m^3 that are considered acceptable, since only one study of this type has been reported [38]. The homogeneity in the number of CFU/m^3 found during the two seasons (rainy and dry) can be explained by the similarity between these season in Mexico City, compared with countries of North America and Europe, where seasons are marked by abrupt changes in ambient temperature and a true separation occurs between intramural and extramural environments, which does not happen in Mexico.

In both hospitals, the fungal diversity was similar, with *Penicillium* spp., *Cladosporium* spp, *A.* section *Nigri*, *A.* section *Flavi,* and *A.* section *Fumigati*, which are all medically relevant, and the most abundant in both hospitals. These results are consistent with those reported by Hao *et al.* [39], who examined the environmental fungal load of two hospitals in China and demonstrated that *Penicillium* spp., *Aspergillus* spp., *Cladosporium* spp., *Alternaria* spp., and *Fusarium* spp. were present in the air, on surfaces, and in the tap water. They also reported that the fungal load fluctuated during the year of air sampling, and the highest densities of these microorganisms were observed during the summer and spring. Azimi *et al.* [40] also found that *Penicillium* spp., *Aspergillus* spp., and *Cladosporium* were the most common genera in different hospitals areas. The diversity of fungi found in this work can be explained because fungi suspended in the atmosphere can be transported quickly as bioaerosols over great distances with the movement of air, which represents the optimal mechanism of dispersion [41]. Certain fungi have developed specialized adaptations that favour their survival and dispersal in the atmosphere, with their transport performed on dust particles, fragments of leaves, skin, clothing fibers, or in drops of water [42]. The physicochemical conditions of the atmosphere do not favour the growth or survival of microorganisms; thus, the majority can only survive in the atmosphere for a short period of time. However, fungal conidia are life forms with longer survival rates and have several properties that contribute to their ability to survive in the atmosphere, such as thick walls, which protect them from desiccation, and pigment (melanin), which aids protects gainst ultraviolet radiation [43]. Additionally, conidia possess other adaptations, such as thermotolerance and nutritional versatility, which allows them to utilize a wide range of carbon and nitrogen sources, as in the case of the genus *Aspergillus* [43]. Also, their hydrophobin-rich conidial external layer allows them to remain suspended in the air without settling, because this cysteine-rich proteins are related to the high surfactant activity of fungi; hydrophobins self-assemble at the hydrophilic-hydrophobic interface to form an amphipathic monolayer [44, 45]. These molecules reduce the surface tension of the medium or substrate on which the fungus grows, allowing it to break the air-water interface and prevent hydrosaturation to maintain gas permeability. The degree of hydrophobicity among fungi ranges from mild to highly hydrophobic, which affects the efficiency of the spore dispersion capacity; for example, conidia from *A. fumigatus* are significantly more hydrophobic than other species in this genus, allowing them to remain in the air [44, 45].

Undoubtedly, the presence of these microorganisms in hospital environments is a warning for more stringent control measures because among these fungi are species of the genus *Aspergillus*, which is considered the main cause of invasive fungal diseases by filamentous fungi in immunocompromised patients, resulting in high mortality rates of 40–90% [46]. Moreover, invasive fungal diseases caused by filamentous fungi other than *Aspergillus* spp. have also increased in frequency and mortality in recent years, especially in immunocompromised patients. The following fungi must be controlled for depending on the susceptibility factors of different hosts: Mucorales, such as *Mucor*, *Rhizopus,* and *Lichtheimia*, *Fusarium*, *Scedosporium*, *Acremonium*, *Penicillium*, *Paecilomyces, Trichoderma,* and dematiaceous fungi, such as *Bipolaris*, *Exophiala*, *Alternaria,* and *Cladosporium* [35]. Invasive fungal diseases caused by these fungi are less common than those caused by the genus *Aspergillus*; however, *Penicillium* spp. can cause certain types of asthma [47], and *Cladosporium* spp. may be associated with changes in lung function in children [48]. In addition, these fungi are usually more virulent and difficult to treat because of their resistance to most of the available drugs and type of affected patient, with those receiving haematologic treatment or solid organ transplants generally more affected [35].

Thermotolerance was conducted at 28 °C and 50 °C to confirm the identification of the *A. fumigatus* section *Fumigati*, because thermotolerance can be used to distinguish *A. fumigatus* from other species of clinical significance belonging to other sections. The *Fumigati* section [49] can be distinguished because of its ability to grow at temperatures of 55 °C and survive at 75 °C, allowing the organism to grow in decaying organic matter and infect mammalian hosts [50, 51]. The results of the presented study revealed that the isolates identified as *A.* section *Fumigati* grew at 28 and 50 °C. However, the fungi identified as *A.* section *Flavi*, *A.* section *Nigri,* and *A.* section *Terrei* only grew at 28 °C. Thus, this phenotypic characteristic is important for confirmation of identification of *A. fumigatus* section *Fumigati* [51].

The GR assays of all the isolates of the genus *Aspergillus* were performed to identify differences which were observed between the GRs of the isolates of *A.* section *Fumigati* at 50 °C. The ability of these fungi to resist high temperatures may play a crucial role in the selection and promotion of pathogenic species, such as *A.* section *Fumigati*, which is a species that adapts to extreme changes in environmental conditions, thus allowing it to develop invasive infections because the microenvironment provided by the human body appears to supply excellent conditions for its growth and invasion. The isolates of *A. flavus* and *A. niger* cannot adapt to similar conditions because they cannot withstand high temperatures [52]. The faster GRs at 28 °C corresponded

to isolates of *A.* section *Nigri*, followed by *A.* section *Flavi*, *A.* section *Fumigati,* and *A.* section *Terrei* from both hospitals. This result was consistent with that of Marin *et al.* [53], who observed that the growth of conidia occurs more rapidly in *A.* section *Nigri* at temperatures between 28–30 °C. However, despite the abundance and high GR of *A.* section *Nigri*, it is less thermotolerant than *A.* section *Fumigati* because its optimal growth temperature is 30 °C. Thus, it is difficult for *A.* section *Nigri* to germinate at 37 °C, which is the temperature of the human body. Perhaps this is a determining factor for why the nosocomial cases of aspergillosis are primarily caused by *A. fumigatus* and not *A. niger.*

The isolation of *A.* section *Terrei* reinforces the findings of Rüping et al. [54] who reported its presence in hospital environments, suggesting that its incidence has increased in cases of IA.

CONCLUSIONS

The airborne fungi in both hospitals was similar, highlighting *Aspergillus* section *Fumigati*, the filamentous fungus responsible for invasive aspergillosis worldwide. The current study presents one of the first to be performed in Mexico for evidence of airborne fungi in nosocomial environments. Furthermore, the isolation, identification and characterization of airborne fungi in hospital environments is a key task, because most of these fungi have the potential to be pathogenic, particularly for patients in intensive care and haematology units. Additionally, the presence of the genus *Aspergillus* within hospital environments should be monitored in areas that house immunosuppressed patients susceptible to exposure to this pathogen. These findings support the necessity to develop stricter safety protocols that would substantially reduce morbidity and mortality in patients and high hospital costs.

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Author´s Contributions
Erick Obed Martínez-Herrera, María Guadalupe Frías-De-León and Esperanza Duarte-Escalante conducted all the experiments. María del Carmen Calderón-Ezquerro, María del Carmen Jiménez-Martínez and Gustavo Acosta-Altamirano, conducted the sampling procedures in the two hospitals. Erick Obed Martínez-Herrera, Facundo Rivera-Becerril and Conchita Toriello conducted statistical analysis, and Facundo Rivera-Becerril and Conchita Toriello provided a critical review of the manuscript. María del Rocío Reyes-Montes was involved in the study design, analysis and interpretation of results and drafted the manuscript. María Guadalupe Frías-De-León and Esperanza Duarte-Escalante contributed to data interpretation and improvement of the manuscript. All of the authors read and approved the final version of the manuscript.

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