Revista Brasileira de Parasitologia Veterinária
Brazilian Journal of Veterinary Parasitology
ISSN 1984-2961 (Electronic)
www.cbpv.org.br/rbpv

Original Article

Cumulative incidence and spatial distribution of dogs exposed to *Toxoplasma gondii*

Incidência cumulativa e distribuição espacial de cães expostos a *Toxoplasma gondii*

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How to cite: Olbera AVG, Fornazari F, Babboni SD, Rossi RS, Sevá AP, Latosinski GS, et al. Cumulative incidence and spatial distribution of dogs exposed to *Toxoplasma gondii*. *Braz J Vet Parasitol* 2020; 29(2): e000820. https://doi.org/10.1590/S1984-29612020025

Abstract

Toxoplasma gondii is one of the most important protozoa parasites worldwide. Although many seroprevalence studies have been performed in domestic and wild species, data on the cumulative incidence and the spatial distribution of *T. gondii* in animals are extremely scarce. In the present study, dogs from Botucatu municipality, São Paulo state, were followed for one year and their blood samples were collected on three moments: days 1, 180, and 360. The sera were submitted to the immunofluorescence antibody test (IFAT) to detect IgG antibodies to *T. gondii*. Age and sex were compared with IFAT results through statistical tests. Spatial analysis was used to detect clusters of seropositive dogs. Among the 350 dogs that were seronegative on day 1, 53 became seropositive in subsequent samplings; thus, cumulative incidence was 15.1% exposed dogs/year. Age and sex were not associated with serological results. The spatial analysis revealed that seropositive dogs were distributed in all the studied areas, with a significant cluster in a zone with poor sanitary conditions and low socioeconomic status. *T. gondii* is frequent and widely distributed in the urban area of Botucatu, and impoverished areas are possibly associated with high levels of environmental contamination by this parasite.

Keywords: Toxoplasmosis, immunofluorescence antibody test, parasitology, IgG, zoonosis.

Resumo

Toxoplasma gondii é um dos parasitas mais importantes mundialmente. Embora muitos estudos de soroprevalência tenham sido realizados em espécies domésticas e selvagens, dados sobre a incidência cumulativa e distribuição espacial de *T. gondii* em animais são extremamente escassos. No presente estudo, cães do município de Botucatu, estado de São Paulo, foram acompanhados por um ano, e suas amostras de sangue foram colhidas em três momentos: dias 1, 180 e 360. O soro foi submetido ao teste de imunofluorescência indireta (RIFI), para detectar anticorpos IgG para *T. gondii*. A idade e o sexo foram comparados com os resultados da RIFI por meio de testes estatísticos. Dos 350 cães soronegativos no dia, 1,53 se tornaram soropositivos nas amostragens subsequentes; portanto, a incidência cumulativa foi de 15,1% de cães expostos/ano. A idade e o sexo não tiveram associação com os resultados da sorologia. A análise espacial demonstrou que os cães soropositivos se distribuíram em todas as áreas compreendidas pelo estudo, com uma concentração significativa em uma região com condições sanitárias precárias e baixos níveis socioeconômicos. *T. gondii* é frequente e amplamente distribuído na área urbana de Botucatu, e regiões empobrecidas podem estar associadas a maiores níveis de contaminação ambiental por esse parasita.

Palavras-chave: Toxoplasmose, reação de imunofluorescência indireta, parasitologia, IgG, zoonose.

Received January 16, 2020. Accepted April 01, 2020.
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Introduction

Few parasites are as prevalent and generalist as *Toxoplasma gondii*. It can infect virtually all warm-blooded species and cause a wide spectrum of health disorders. Species of the Felidae family are the definitive hosts, contaminating the environment with parasitic oocysts that are eliminated through their feces. The main transmission routes of *T. gondii* to its hosts are ingestion of oocysts present in water, food, and soil, ingestion of raw or undercooked meat from infected animals, and transplacental transmission. Although most infections are asymptomatic or self-limiting, toxoplasmosis can have a great impact on human public and animal health depending on the parasite genotype, inoculum, the species infected, and the immune status of the host (Dubey, 2010; Calero-Bernal & Gennari, 2019).

Domestic dogs can serve as sentinels for human parasites (Salb et al., 2008; Pinto-Ferreira et al., 2019). Therefore, the rate of *T. gondii* exposure in dogs can indicate the level of environmental contamination by this parasite and, therefore, the risks of exposure to humans and other animal species (Yan et al., 2012). A recent study in Brazil demonstrated that dogs may not be a good indicator of *T. gondii* exposure for their respective owners, but they can be useful sentinels for environmental infection and outbreaks (Benitez et al., 2017). Although many seroepidemiological surveys have been performed in dogs, most of them consist of transversal (prevalence) studies. The cumulative incidence, however, has been poorly assessed. In the present context, cumulative incidence can be defined as the number of seropositive (exposed) animals over a specific period of time, which provides valuable information on the exposure to pathogens from a chronologic viewpoint. Such assessment is hard to execute mainly owing to the difficulties in sampling animals more than once and following them for long periods. But considering the valuable data obtained from longitudinal surveys, we believe they should be more encouraged. Hence, these remarks led us to conceive the present study.

The aims of the present study were to access the cumulative incidence (hereafter referred to as incidence) and the spatial distribution of dogs exposed to *T. gondii* through detection of IgG antibodies. Additionally, the association of age and sex with the incidence results and the prevalence of exposure in different sampling moments were evaluated.

Material and Methods

Dogs and samples

Samples from a previous longitudinal study conducted by Babboni et al. (2014) were used. Dogs were followed for 360 days, and their blood samples were collected at three stages with 180-days intervals. All dogs were from Botucatu municipality, located in the center of São Paulo State, which is the most populated and economically developed state of Brazil. Botucatu has a population of approximately 140,000 habitants, annual rainfall average of 1,358 mm, and annual mean temperature of 20.7ºC (Fornazari et al., 2018).

Dogs were first sampled during the annual vaccination campaign against rabies performed by the Environmental Health Surveillance Department of Botucatu. The animals were randomly chosen in 13 vaccination stations distributed in the five regions of the city (North, South, West, East, and Central). Stations with the highest number of dogs were chosen to optimize the sampling. Only apparently healthy dogs were included.

Blood was taken from 576 dogs after getting the owner's consent, and the serum samples obtained were stored at -20ºC. Subsequent samples were taken during home visits. Three serum samples were analyzed: day 1 (1^{st} sampling), day 180 (2^{nd} sampling), and day 360 (3^{rd} sampling). Age, sex, and residential address were recorded for each animal. By the end of the study, it was not possible to collect subsequent samples from all of the dogs because many of them had died or moved from the residence, or the owner was no longer participating in the study, among other reasons. Three serum samples were successfully obtained from 369 dogs, which were included in the survey.

The study was approved by the Ethics Committee on Animal Use (CEUA) from the School of Veterinary Medicine and Animal Science (FMVZ), São Paulo State University (UNESP), under protocol n. 64/2009. The work was carried out in accordance with the Directive 2010/63/EU on the protection of animals used for scientific purposes.

Laboratory tests

Detection of IgG antibodies to *T. gondii* was considered evidence of exposure to *T. gondii* and was assessed through an in-house immunofluorescence antibody test (IFAT). Serum samples were diluted in phosphate buffered saline pH 7.2 (PBS) at 1:25 dilution, and then 10 µl were placed in microscopy slides previously prepared with tachyzoites of *T. gondii* (RH strain). Parasites were produced in the laboratory's own premises, using 30-day old female Swiss mice. Slides were incubated at 37°C for 30 minutes and then washed twice with PBS. A fluorescent secondary antibody (anti-dog IgG), provided by the Zoonosis Control Center of São Paulo, was added to the slides. Incubation and washing steps were performed one more time and the slides were visualized under a fluorescence microscope (Axio Imager A2, Carl Zeiss). Results were considered to be positive when tachyzoites showed intense fluorescence and when they were evaluated by two or more persons to avoid interpretation biases. Positive samples were further tested in two-fold dilutions until the final titer. There is no standard cut off titer for the IFAT in dogs, and it can range from 1:8 to 1:200 (Dubey, 2010). We adopted 1:25 as a reasonable cut off titer, since it is frequently used in several animal species and serological tests.

Data analysis

Incidence of exposure was calculated through detection of new cases during the study period. A new case was any dog that was seronegative on day 1 and became seropositive on day 180 or on day 360 (or both). The 19 dogs that were already seropositive in day 1 (see Results) were excluded from the incidence calculation, and the remaining 350 seronegative dogs were used. The entire study period comprised 360 days, which was considered as one year. Thus, the incidence was expressed as the number of exposed dogs/year.

The sex and age of the dogs were compared to the incidence of new exposures using the chi-square test and the Fisher's exact test. Age was recorded in three categories: 1) between two months and one year; 2) between one and five years; and 3) more than five years. Age was not recorded for three animals, and they were excluded from the analyses with an age variable. The relationship between incidence and age was tested for males only, females only, and both males and females to explore possible confounding factors. Tests considered 5% as the significance level.

Prevalence was defined as the ratio of the number of seropositive dogs to the total number of dogs sampled and was calculated for each of the three samples. All combinations between serological results and the three samples (negative/positive X day 1/180/360) were considered, and the respective frequencies were calculated. The serological titers obtained in the IFAT in different samples were compared through their means and quartiles using a boxplot.

All the frequencies had 95% confidence intervals estimated using the binomial distribution. All data analyses and statistical tests were performed using R v.3.6.1 and SAS v.9.4 software.

Spatial analysis

The residences of each animal were georeferenced using the Google Maps service for subsequent analysis. Two types of spatial analyses were performed: clustering and kernel density estimation. The origins of four dogs were not recorded, and they were excluded from these tests.

Local cluster analyses were performed to assess the spatial and temporal prevalence of dogs to identify areas with potential of *T. gondii* transmission. The spatial scan statistics method was applied as described by Kulldorff & Nagarwalla (1995) using the SatScan™ 9.5 software. Seropositive and seronegative dogs were considered as cases and control, respectively, for calculating the local rates inside varied sizes of circles through statistical modeling using *Bernoulli* distribution. For each possible cluster, the likelihood-ratio test was calculated by comparing the hypothesis that the risk of being positive is greater within the circle against the hypothesis that the risk is equal for both inside and outside the circle. The cluster was calculated separately for each sample with a significance level of 5%. The circle with the maximum value of the likelihood ratio was considered the most probable cluster (Kulldorff, 1997; Wheeler, 2007; Pfeiffer et al., 2008), and it was represented on the map using QGIS v.3.4 software.

Kernel density estimation was performed for both seropositive and seronegative dogs in order to visualize the spatial distribution and intensity during the entire study period (Pfeiffer et al., 2008). All the three samples were combined into a single group of data; thus, each dog was represented three times in the map. The kernel quartic function was used to create a smooth spatial surface in the QGIS v.3.4 software, which was also used to create the maps.

Results

Among the 350 dogs that were seronegative in the beginning of the study (day 1), 53 became seropositive in subsequent samples. Thus, the incidence of exposed dogs was 15.1%/year (53/350; CI 95% 11.5 – 19.3). Associations of seropositive dogs with age and sex were not significant (Table 1). The prevalence of positive dogs in the 1st, 2nd, and 3rd samples were 5.1%, 6.5%, and 15.1%, respectively (Table 2). Serologic titers ranged from 25 to 400, with higher titers being more frequent in the 3rd sample (day 360). However, the differences in titer values between samples were not statistically significant (data not shown). All possible outcomes by combining the results of the three samples are shown in Table 3, as well as the respective number of animals.

Table 1. Association between age and sex with incidence of exposure to *T. gondii* in dogs from Botucatu municipality, Brazil.

Variable		Incidence of exposure				P-value
		Positive[a]	Negative[a]	%	CI 95%	
Sex	Female	26	152	15.0	10.1-21.1	0.974
	Male	27	145	15.2	10.1-21.4	
Age	2 months to 1 year	43	248	14.7	10.9-19.3	0.102
	1 to 5 years	8	45	17.7	6.7-27.6	
	> 5 years	0	3	0	0.3-1.0	
	unknown	2	1	-	-	
Total		**53**	**297**	**15.1**	**11.5-19.3**	

[a] Number of animals; CI: Confidence interval.

Table 2. Prevalence and titers of dogs seropositive to *T. gondii* in Botucatu municipality, Brazil.

Sampling moment (day)	N^P	N^T	Prevalence (%)	CI 95%	Titers*				
					25	50	100	200	400
1	19	369	5.1	3.7-7.9	8	8	0	2	1
180	24	369	6.5	4.2-9.5	8	10	2	4	0
360	56	369	15.1	11.6-19.2	21	13	10	6	6

N^P: Number of positive animals; N^T: Number of animals tested; CI: Confidence interval; *Number of animals for each serological titer.

Table 3. Serological results according to sampling moment in dogs tested for *T. gondii* in Botucatu municipality, Brazil.

Number of dogs	%	Results according to sampling moment*		
		1st sampling (day 1)	2nd sampling (day 180)	3rd sampling (day 360)
296	80.22	-	-	-
4	1.08	-	+	-
8	2.17	-	+	+
42	11.38	-	-	+
6	1.63	+	-	-
7	1.9	+	+	-
5	1.36	+	+	+
1	0.27	+	-	+
369	**100**	**19**	**24**	**56**

* negative (-), positive (+).

The kernel map showed that seronegative dogs were distributed in nine main regions of the city in high intensity, excluding the west area (Figure 1). Areas with high intensity of seropositive dogs were also the areas with high intensity of seronegative ones. The seroprevalence was significant in the 3rd sampling (Figure 2), with a cluster of 28.9% of prevalence and relative risk of 4.6 in the south west region of the city. No clusters of seropositive dogs were detected in the 1st and 2nd samples.

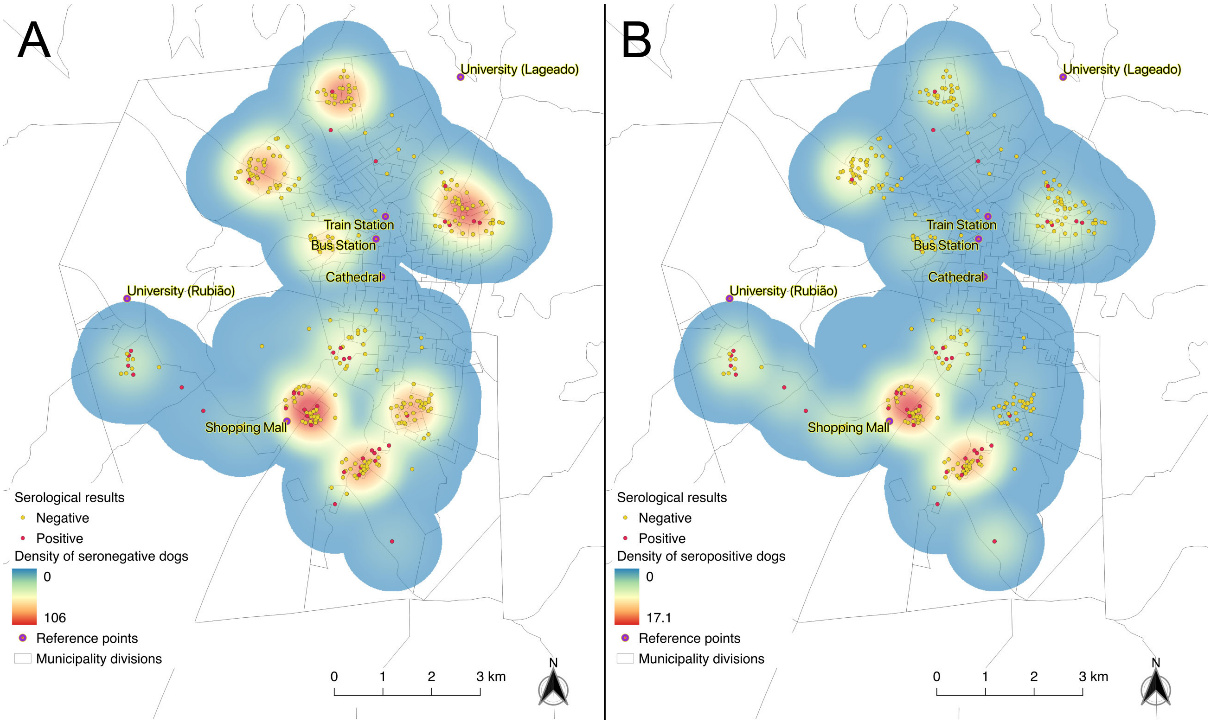

Figure 1. Kernel density estimation of dogs seronegative (A) and seropositive (B) for *T. gondii* in Botucatu municipality, Brazil.

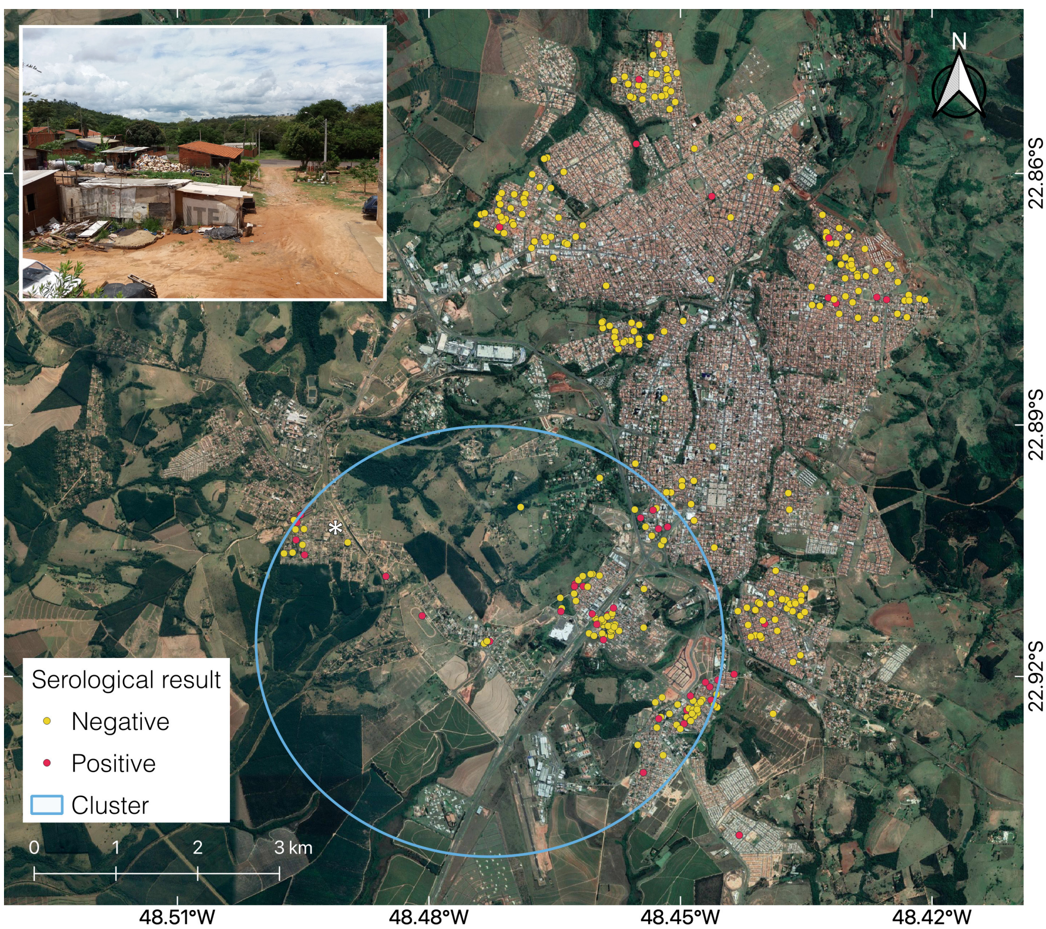

Figure 2. Cluster analysis of dogs seropositive for *T. gondii* in Botucatu municipality, Brazil (cluster radius: 2.85 km; population: 124; positive cases: 37; frequency of positive cases: 29.8%; expected positive cases: 11.2%; observed/expected positive cases: 3.3; relative risk: 4.6; P value: 0.000000037). White asterisk indicates the location of the picture in the upper left corner.

Discussion

The incidence indicated that a significant number of dogs became exposed over the studied period. It is difficult to compare these results with those from the literature, as longitudinal surveys in animals are extremely scarce. To the best of our knowledge, this study is one of the first assessments of *T. gondii* incidence in dogs. One of the few prospective studies in animals reported a similar rate, with 17% seroconversion/year in an urban population of stray cats in France (Afonso et al., 2006). Nevertheless, the 15.1% incidence rate in Botucatu demonstrates the dynamic of *T. gondii* exposure in dogs, providing new and quantitative data on the epidemiology of this parasite in the urban area of a mid-sized Brazilian city. This result indicates the frequent presence of *T. gondii* in the urban environment, and highlights the risks of infection to humans and other animal species.

The sex of the dogs was not associated with positive results in serological tests. Many studies corroborate our findings (Ali et al., 2003; Azevedo et al., 2005; Yan et al., 2012; Langoni et al., 2013; Gao et al., 2016). The gender of many animal species can influence the susceptibility to infections and diseases. Males are usually more susceptible than females, which is probably associated with immunosuppressive effects of androgen hormones and to disease resistance genes that are affected by steroid hormones (Klein, 2000). However, these factors may not influence the serological response of dogs exposed to *T. gondii*, as supported by our results and the scientific literature.

Age was not statistically significant among seropositive dogs. The literature still has no consensus on this topic, as there are several studies both confirming and contradicting that age is related to the risks of exposure to *T. gondii*. This ambiguity may be related to the complex epidemiology of this parasite across different scenarios worldwide. However, there is substantial evidence that older dogs have more chances of becoming seropositive (Cañón-Franco et al., 2004; Azevedo et al., 2005; Camossi et al., 2008; Langoni et al., 2013; Gao et al., 2016). According to these studies, the probable explanation is that older dogs have more chances of exposure to the parasite over time than younger ones. This hypothesis is highly plausible considering the chronic nature of *T. gondii* infection.

The rate of positive dogs among prevalence studies varies greatly. In Brazil, seroprevalence in dogs can range from 3.1% to 91% in different regions (Dubey et al., 2012). Such disparities reflect the epidemiology of different settings and the methodology of each study, such as sampling design, laboratory tests, study location, among other factors. The prevalence presented here relates to a large-sized sample, including randomly selected dogs that are widely distributed in an urban area. It is possible that the inclusion of more dogs above five years old would increase the rate of seropositivity. In this case, the incidence and prevalence would be higher than we detected. The predominance of young dogs in the population surveyed was a consequence of using animals from the vaccination campaign, which caters to many low-income communities where the life expectancy of dogs tends to be low.

The kernel maps showed that both seropositive and seronegative dogs were widely distributed in the urban area of Botucatu, indicating the extension of the animal population studied. Although seropositive dogs were more concentrated in the south area, they were present in all nine heat areas (Figure 1), demonstrating that *T. gondii* is geographically dispersed in the city. Although kernel maps are very useful for illustration purposes, they should be carefully interpreted as they do not compare positive and negative cases.

A significant cluster of seropositive dogs was identified in the southwest region of the city. The concentration of cases in this area is possibly associated to the low socioeconomic status of the population and to deficient infrastructure, conditions that may increase the exposure to several environmental pathogens. According to the Environmental Health Surveillance Department of Botucatu, this cluster is present in the most impoverished area included in the study – although there are no official data available to corroborate this observation. Nonetheless, this area is inhabited by many low-income families, stray dogs and cats, environmental pollution by domestic garbage, and, in some places, absence of impermeable soil (Figure 2). In 2006, our research team observed a high seroprevalence (56%) in dogs surveyed exclusively in this zone (Santa Elisa neighborhood) (Camossi et al., 2008), corroborating the present findings. Thus, the area inside the cluster probably has higher levels of environmental contamination by *T. gondii* than the areas outside the cluster. These data can assist public health authorities to guide future investigations regarding toxoplasmosis in Botucatu, as well as activities directed toward prevention and control of this important disease.

Conclusions

This study detected a cumulative incidence of 15.1% of exposed animals/year by *T. gondii* among dogs inhabiting the urban areas of a mid-sized Brazilian city, providing quantitative data on the epidemiology of this important zoonotic parasite. Seropositive dogs were concentrated in the south-west region of Botucatu, which is characterized by deficient infrastructure and the low socio-economic status of the population. These results demonstrate that *T. gondii* is frequent and geographically widespread in the urban area of Botucatu, and that impoverished zones are possibly associated with high levels of environmental contamination by *T. gondii*.

Acknowledgements

We are thankful to São Paulo Research Foundation (FAPESP, process nº 2016/17503-3) for the financial support.

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