











Prevalence and Risk Factors of Pulmonary Fungal Pathogens among Symptomatic Patients with or without Tuberculosis at Gombe, Nigeria

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ABSTRACT

Introduction: Pulmonary fungal infections are a significant etiology of morbidity among immunocompromised and immunosuppressed patients. This study aimed to determine the prevalence of fungal pathogens and associated risk factors among pulmonary tuberculosis (PTB) and non-PTB patients attending Federal Teaching Hospital, Gombe, Nigeria. **Methods:** Three consecutive early morning sputum samples were collected from 43 PTB patients and 173 non-PTB persons and then examined for fungal pathogens using standard mycological stains, microscopy, and biochemical assays. All the participants were screened for HIV by the World Health Organization HIV testing algorithm and *M. tuberculosis* infection using GeneXpert® nested PCR equipment. Samples with at least two significant fungal growths were considered positive. **Results:** Out of 216 sputa, 73.6% showed fungal growth in cultures. One hundred percent and 67% of PTB and non-PTB participants had positive sputa culture, respectively. In PTB patients, *Candida albicans* (25.6%) and *Aspergillus fumigatus* (20.9%), and in non-PTB individuals *A. fumigatus* (51.7%) and *A. niger* (17.2%) were the most prevalent species. Age and residential areas were significantly associated with fungal infection in PTB and non-PTB subjects ($p < 0.05$). Cigarette smoking, prolonged antibiotic use, and having domestic pets were significant risk factors for developing pulmonary fungal infections in both groups ($p < 0.05$). None of the studied risk factors was significantly associated with pulmonary mycosis among TB patients ($p > 0.05$). However, prolonged use of antibiotics was a significant risk factor of pulmonary fungal infection among non-TB patients ($p = 0.009$). **Conclusion:** Our study showed that PTB was a predisposing factor for fungal infection, especially among individuals with low socioeconomic status.

INTRODUCTION

Due to the ubiquitous nature of fungal spores and yeast, humans regularly inhale them [1]. These microorganisms readily are deposited in the airways, and some reach the alveolus due to their small sizes. In immunocompetent persons with healthy lungs, fungal spores usually are eliminated by innate immune defenses [2]. However, in patients who suffer from long-term respiratory tract

disorders such as chronic obstructive pulmonary disorder, pulmonary tuberculosis (PTB), and cystic fibrosis, fungal spores could easily evade the lung immune apparatuses, germinate and colonize in the respiratory tract [3]. Consequently, the persistence of fungal pathogens in these patients' pulmonary tract predisposes them to develop chronic and systemic fungal diseases such as invasive pulmonary aspergillosis [4].

Some of the inhaled fungi species can thrive at body temperature and evade healthy persons' host defensive mechanisms [5]. However, very few fungi that cause lung infection are true pathogens, most notably, those that cause endemic mycoses. Six members of the genera *Aspergillus*, *Candida*, and *Cryptococcus*, can overwhelm innate immunity when inhaled in large quantities or when patients become immunodeficient or immunosuppressed [5, 6].

Globally, PTB caused by *Mycobacterium tuberculosis* is among the first ten causes of death [7]. About 95% of PTB cases live in developing countries in Africa and Asia due to inadequate diagnostic equipment and a lack of access to chemotherapeutic drugs [8]. In 2018, Nigeria alone recorded 103,921 new and relapse PTB cases [9]. Progression of PTB, deterioration of lung structure and function, prolonged use of anti-TB, and immunosuppressive drugs make PTB patients vulnerable to fungal lung infections. When the host defense mechanism deteriorates, coinfections with opportunistic fungi may significantly affect the prognosis of PTB and even result in death [10].

Fungal infections are estimated to kill over 1.5 million and affect >1 billion people worldwide [10]. These infections are neglected by most health care systems, even though most fatalities from fungal diseases are preventable. Prompt and accurate diagnosis of fungal infections provides clinicians with the opportunity to adopt appropriate antifungal therapy. However, delayed or unavailable diagnosis and appropriate antifungal treatments in most low and middle-income countries lead to severe chronic illness or even death [11]. Recent global data reported ≈3,000,000 cases of chronic pulmonary aspergillosis, ≈223,100 cases of cryptococcal meningitis, especially in people with HIV/AIDS, and ≈700,000 cases of invasive candidiasis [11].

A paucity of information on the incidence and prevalence of pulmonary fungal infections in many low- and middle-income nations has led to an underestimation of their real public health significance [12]. The present comparative cross-sectional study aimed to determine the prevalence and profile of fungal pathogens and associated risk factors among PTB and non-PTB individuals attending Federal Teaching Hospital, Gombe, Nigeria.

MATERIAL AND METHODS

Study area. This study was conducted at the Federal Teaching Hospital Gombe (FTHG) in the city of Gombe, the capital of Gombe state. Gombe, in the center of North-East of the country, is one of the 36 states of the Federal Republic of Nigeria. It has a population of ≈2,857,042 and covers an area of 20,265 km². The temperature averages 30°C with an annual rainfall of 1200 mm. The predominant occupations of people are agriculture and livestock rearing.

Ethical Consideration. This study was approved by the Human Research Ethical Committee (NHREC/25/10/2013) of the Federal Teaching Hospital, Gombe State, Nigeria. Written informed consent was obtained from participants or their guardians following the standards of the Helsinki Declaration of 1975 (revised in 2000). The generated data were analyzed anonymously throughout the study.

Methodology. This comparative cross-sectional study began on 02 May 2017 and ended on 30 May 2018. The minimum sample size calculated using the Fischer expression for cross-sectional studies based on a previous prevalence of 13.5% pulmonary fungal infection [13] was 179; however, we increased it to 216 participants.

Individuals presenting with both respiratory and constitutional symptoms similar to that of pulmonary tuberculosis (such as fever, weight loss, malaise, chest pain, and persistent cough) with no history of diabetes mellitus and other immunosuppressive diseases were enrolled, and healthy individuals with no clinical symptoms of pulmonary tuberculosis or having diabetes mellitus and other immunosuppressive diseases such as cancer were excluded from the study.

The participants were outpatients, aged 15-50 years, in the general clinics of FTH Gombe. They were referred to the Department of Medical Microbiology and Immunology of FTH Gombe for the microscopic examination of their sputum for acid-fast *bacilli*. We explained the study to all participants and educated them on accurately collecting sputum samples to avoid contaminations. Three consecutive early morning sputum samples in the sterile, wide neck, leak-proof containers, and 2 ml of blood were collected from the participants. Sociodemographic data and risk factors, including antibiotics use, having pets, and cigarette smoking was registered in a structured questionnaire.

***Mycobacterium tuberculosis* nested PCR.** Sputum samples were mixed with the PCR reagents provided by the manufacturer (Cepheid GeneXpert, California, USA). The mixtures were transferred on a cartridge and placed in the GeneXpert machine (Cepheid, California, USA). From this point on, all processing was fully automated following the manufacturer's instruction (Cepheid®, CA, USA). The test simultaneously detects *Mycobacterium tuberculosis* complex (MTBC) and resistance to rifampin (RIF) in less than 2 h.

HIV Serological Test. The participants' sera were examined for HIV using rapid tests Determine™ (Alere, Auckland City, New Zealand), Uni-Gold™ TM Recombigen®HIV-1/2 (Trinity Biotech, Wicklow, Ireland) and Chembio HIV 1/2 STAT-PAK® (Medford, New York, USA) as instructed by manufacturers.

Microscopic Examination of Sputum. We used potassium hydroxide (KOH) test and Gram staining to detect yeasts and fungi, and Giemsa staining for cyst and

trophozoite of *Pneumocystis jirovcii* as previously described by others [14]. Simply, a small portion of the sputum was mixed with a drop of 10% KOH on a clean, grease-free glass slide. The preparation was flattened under a coverslip, placed in a moist chamber, and kept at room temperature for 30 min, and then examined for the presence of fungal elements by microscopy with 100X and 40X magnifications [14].

Isolation of Fungal Species. A loopful of the sputum sample was streaked on the surface of the sterile Sabouraud Dextrose Agar (SDA) containing chloramphenicol (Art. No. 06-118CASE) using a sterile wire loop [16] and incubated at ambient temperature and 37°C for 7 to 14 days, as described previously [15].

Characterization of Isolates. The fungal isolates were characterized using standard mycological procedures, including colony morphology, physiological, and biochemical tests.

Molds were identified based on colony morphology, i.e., growth rate, surface texture, and pigmentation. The yeast-like colonies were identified based on germ tube production, chlamydospore formation on cornmeal agar, sugar fermentation tests, and temperature studies using malt extract agar [14].

Characterization of Yeasts Isolates on Corn Meal Agar. The yeast isolates were cultivated on cornmeal agar with Tween 80 and then differentiated based on examining structural differences, e.g., production of pseudohyphae, blastochonidia, and chlamydospores by microscopy [14].

Sugar Assimilation Test. Six sugars, including glucose, maltose, sucrose, lactose, xylose, inositol, were utilized for these purposes, and gas production was defined as positive for fermentation. This test determines the ability of a yeast isolate to use a particular carbohydrate substrate as its sole carbon in a medium. For this, yeast nitrogen base medium containing 1g KH₂PO₄, 0.5g MgSO₄, 5g NH₄SO₄, 25g Agar-Agar, and distilled water to final volume 1L was prepared and autoclaved at 121°C for 15 min. Yeast suspensions were prepared in a 2ml yeast nitrogen base by adding heavy inoculums from the cultures. The suspensions were added to the 18ml of molten agar (cooled to 45°C), mixed well, and poured into a 90 mm Petri plate. The plates were allowed at room temperature harden, and then the carbohydrate impregnated discs were placed onto the surface of the agar plate. The plates were incubated at 37°C for 3-4 days. Glucose was used as a positive control because all the *Candida* species assimilate this carbohydrate. The presence of a halo of growth around each carbohydrate indicated carbohydrate assimilation [17].

Sugar Fermentation Test. Liquid fermentation medium containing peptone 1%, sodium chloride 0.5%, Andrade's indicator 0.005% was autoclaved at 121°C for 15 min, and then added with a filter-sterilized sugar at

the 2% concentration. The medium was divided into the sterile test tubes at 5 ml volumes, followed by a Durham tube inserted into each tube and plugged with cotton. The inocula were prepared by suspending a large quantity of yeast on a sugar-free medium. Each carbohydrate broth was inoculated with approximately 0.1ml of the prepared inoculum. The tubes were incubated at 25°C and examined every 48 h for a week for the production of acid (pink color) and gas in Durham tubes. Production of gas in the tube was considered as fermentation [17].

Temperature for Isolation and Growth. This method determines the ability of *Candida* spp. in growing at elevated temperatures. The *C. albicans* and *Candida tropicalis* were streaked on the surface of malt extract slant and incubated at 37°C and 25°C. The tubes were examined daily for up to 7 days for the growth of the isolates. Molds were identified based on their colony morphology, such as surface topography, surface texture, pigmentation macro- and micro-morphology [17]. Identification of *Candida guilliermondii*, *Penicillium glabrum*, and *Penicillium citrinum* to species level was based on macroscopic morphology on SDA culture, and microscopic features as previously described [17].

Statistical Analysis. Data obtained were entered in Microsoft Excel sheet and statistically analyzed using statistical package for social sciences (SPSS) software version 24.0. Two-tailed Chi-square was used to determine the association between two categorical variables. Bivariate logistic regression was used to determine the Odds Ratios (OR) of contracting pulmonary fungal infections. *P* values ≤ 0.05 at 95% confidence intervals were considered significant.

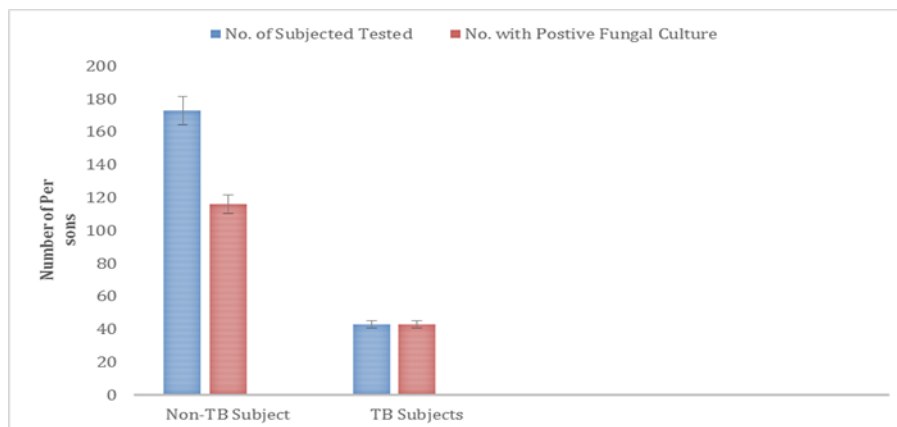
There was a significant association between the prevalence of pulmonary fungal infection with participants' PTB status ($\chi^2=23.04.15$; $p<0.0001$). Age and residential areas were significantly associated with fungal infection in both PTB and non-PTB subjects ($p<0.05$), but socioeconomic status and level of education were not significantly associated with PTB-fungal coinfection ($p>0.05$) (Table 2).

RESULTS

Out of the 216 participants, 159 (73.6%) showed fungal cultures in sputum, including (100%) of PTB and 67% non-PTB participants (Fig. 1). *C. albicans* (25.6%) and *A. fumigatus* (20.9%) in PTB patients and *A. fumigatus* (51.7%) and *A. nigar* (17.2%) in non-PTB participants were the most prevalent fungal species (Table 1). There was a significant association between the prevalence of pulmonary fungal infection with PTB status of participants ($\chi^2=23.04.15$; $p<0.0001$). Age and residential areas were significantly associated with fungal infection in both PTB and non-PTB subjects ($p<0.05$), but socioeconomic status and level of education were not significantly associated with PTB-fungal coinfection ($p>0.05$) (Table 2).

Table 1. Distribution Pattern of Pulmonary Fungal Isolates from Persons attending Federal Teaching Hospital Gombe

| Fungal Isolates | No. (%) in PTB | No. (%) in non-PTB |
|-------------------------------|----------------|--------------------|
| <i>Aspergillus fumigatus</i> | 9 (20.9) | 60 (51.7) |
| <i>Aspergillus niger</i> | 8 (18.6) | 20 (17.2) |
| <i>Aspergillus flavus</i> | 2 (4.6) | 0 (0.0) |
| <i>Candida albicans</i> | 11 (25.6) | 15 (12.9) |
| <i>Candida guilliermondii</i> | 4 (9.3) | 4 (3.4) |
| <i>Candida tropicalis</i> | 7 (16.3) | 8 (6.9) |
| <i>Penicillium glabrum</i> | 0 (0.00) | 3 (2.6) |
| <i>Penicillium citrinum</i> | 2 (4.6) | 3 (2.6) |
| <i>Mucor spp</i> | 0 (0.0) | 3 (2.6) |
| Total | 43 (100) | 116 (100) |

**Fig 1.** Comparative Prevalence of Fungal Isolates among TB and non-TB patients. Df = 2, $\chi^2=23.04.15$; $p<0.0001$

Regarding the risk factors associated with the pulmonary fungal infection, 100% of individuals on prolonged antibiotic use of ≥ 3 weeks ($n=60$) showed a pulmonary fungal infection, while the rate among those with no antibiotic therapy ($n=95$) was 60.9%. Participants who had household pets showed a relatively higher chance of acquiring a pulmonary fungal infection; 100% ($n=33$) vs. those kept no pets 68.9% ($n=126$). Cigarette smokers included higher pulmonary fungal infection cases than nonsmokers, 100% ($n=19$) vs. 71.1% ($n=140$).

Univariate logistic regression analysis of all subjects showed that cigarette smoking (OR=15.9 [95% CI: [0.9-268.8]), prolong antibiotic use (OR=77.9 [95% CI: 77.9 (4.7-1283)]) and possession of domestic pet (OR= 77.9 [95% CI:1.8-505.7]) were significant risk factors associated with pulmonary fungal infections in both groups ($p<0.05$) (Table 3a). However, with separate univariate analysis, none of the studied risk factors was significantly associated with pulmonary mycosis in TB patients ($p>0.05$) (Table 3b). However, prolonged use of antibiotics was a significant risk factor for pulmonary fungal infection among non-TB patients ($p=0.009$) (Table 3c).

DISCUSSION

Pulmonary fungal infections constitute the leading cause of morbidity and mortality among immunocompromised patients with TB and HIV and those on prolonged antibiotic and steroid use [18]. The pulmonary mycosis infections are misdiagnosed and underdiagnosed due to the non-specific clinical presentations of the infection [19, 20, 21]. Thus, there is a need to properly evaluate and diagnose the fungal pathogens, especially in persons presenting with symptoms similar to PTB.

To the best of our knowledge, this is the first study in Northern Nigeria on the prevalence of pulmonary fungi and tuberculosis coinfection. In our study, fungal cultures were observed in 73.6% of participants, including 100% ($n=43$) and 67% ($n=116$) of PTB and non-PTB individuals, respectively. Our results showed a significantly higher pulmonary fungal infection rate than previous studies [7, 22, 23]. This variation in the prevalence might be due to differences in geographical locations, the national burden of fungal infections, sample size, study design, type of culture media, and laboratory technique [23].

Table 2. Prevalence of Pulmonary Fungal Infection by Sociodemographic variables of all Subjects

| Variables | Non-TB Subjects | | <i>p</i> -value | TB Subjects | | |
|-----------------------------|-----------------|------------------|-----------------|-------------|------------------|-----------------|
| | No. of Subjects | No. (%) Positive | | No. tested | No. (%) Positive | <i>p</i> -value |
| Age | | | 0.0009* | | | 0.003* |
| ≤14 | 42 | 32 (76.2) | | 9 | 9(100) | |
| 14 – 30 | 69 | 35 (50.7) | | 12 | 12(100) | |
| >30 | 62 | 49 (79.0) | | 22 | 22(100) | |
| Sex | | | 0.1272 | | | 0.647 |
| Male | 88 | 62 (70.5) | | 20 | 20(100) | |
| Female | 85 | 54 (64.5) | | 23 | 23(100) | |
| Residence | | | <0.0001* | | | 0.004* |
| Urban | 74 | 68 (91.9) | | 31 | 31(100) | |
| Rural | 99 | 48 (48.5) | | 12 | 12(100) | |
| Level of Education | | | 0.4632 | | | 0.0027* |
| No formal | 38 | 24 (63.2) | | 18 | 18(100) | |
| Primary | 49 | 36 (73.5) | | 13 | 13(100) | |
| High School | 35 | 20 (57.1) | | 6 | 6(100) | |
| College | 51 | 36 (70.6) | | 6 | 6(100) | |
| Marital status | | | 0.632 | | | 0.446 |
| Not Married | 79 | 53 (67.1) | | 19 | 19(100) | |
| Married | 94 | 63 (67.0) | | 24 | 24(100) | |
| Socioeconomic Status | | | 0.358 | | | 0.0003* |
| Low | 76 | 54 (71.1) | | 26 | 26(100) | |
| Middle | 34 | 21 (61.7) | | 12 | 12(100) | |
| High | 63 | 41 (65.1) | | 5 | 5(100) | |

*Significance determined by 2 tailed Chi-squared test

In the present study, we observed yeasts in 51.2%, and filamentous fungi in 48.8% of samples. Our results are in agreement with the Kalyan *et al.* (2016), who reported 54.8% and 45.2% of yeasts and filamentous fungi, respectively [18], but different from another study

reporting 91.6% *Candida* spp. and 8.4% *Aspergillus* spp. infections [23]. The predominant isolated filamentous fungus in the present study was *A. fumigatus*, similar to other studies [10, 24].

Table 3a. Risk Factor analysis of Pulmonary Fungal Infection among all Subjects

| Characteristics | No. of persons tested | No. (%) positive | OR (95% CI) | <i>p</i> value |
|--|-----------------------|------------------|------------------|----------------|
| Prolong antibiotic use (≥3 weeks) | | | | |
| Yes | 60 | 60 (100) | 77.9 (4.7-1283) | 0.002* |
| No | 156 | 99 (60.9) | | |
| Total | 216 | 159 (73.6) | | |
| Possession of Pets | | | | |
| Yes | 33 | 33 (100) | 30.5 (1.8-505.7) | 0.017* |
| No | 183 | 126 (68.9) | | |
| Total | 216 | 159 (73.6) | | |
| Cigarette Smoking | | | | |
| Yes | 19 | 19 (100) | 15.9 (0.9-268.8) | 0.05* |
| No | 197 | 140 (71.1) | | |
| Total | 216 | 159 (73.6) | | |

*Significance determined by Bivariate Logistic Regression

There are reports of the relationship between fungus and tuberculosis infection [7, 8, 25]. Here, we found fungal infection among 100% PTB cases. This rate is very high compared to previous reports of 12.3% and 24% prevalence of positive fungal culture from PTB patients [7, 22]. This difference could be due to poor personal and environmental hygiene of PTB patients and the endemicity of fungal pathogens in the study area. The present study revealed a significant association between the prevalence of pulmonary fungal pathogens with PTB ($p < 0.0001$), which might be related to the compromised immune status of the subjects. PTB patients are believed to experience a

particular form of immunosuppression and are at high risk of contracting secondary mycological agents.

Opportunistic fungal infections are usually capable of causing disease in immunocompromised patients and those with prior lung disorders [25, 26]. However, these pathogens are usually asymptomatic or self-limiting in immunocompetent persons. In our study, 79% of non-TB patients had fungal infections. This may be because most of them were on antibiotic self-medication, which might have facilitated the proliferation of fungal pathogens [27, 28]. Indeed, disturbance of the respiratory bacterial flora is a risk factor for fungal colonization and infections [29, 30].

Table 3b. Risk Factor analysis of Pulmonary Fungal Infection among TB Subjects

| Characteristics | No. of persons tested | No. (%) positive | OR (95% CI) | p value |
|---|-----------------------|------------------|-------------------|---------|
| Prolong antibiotic use (≥ 3 weeks) | | | | |
| Yes | 30 | 30 (100) | 2.26 (0.04-119.9) | 0.688 |
| No | 13 | 13 (60.9) | | |
| Total | 43 | 43 (73.6) | | |
| Possession of Pets | | | | |
| Yes | 30 | 30 (100) | 2.26 (0.04-119.9) | 0.688 |
| No | 13 | 13 (68.9) | | |
| Total | 43 | 43 (73.6) | | |
| Cigarette Smoking | | | | |
| Yes | 10 | 10 (100) | 2.26 (0.04-119.9) | 0.688 |
| No | 33 | 33 (71.1) | | |
| Total | 43 | 43 (73.6) | | |

*Significance determined by Bivariate Logistic Regression

Most studies showed higher rates of fungal infections in the old age groups and a correlation between an increase in age and *Aspergillus* and Tuberculosis coinfection [7, 16]. These reports are in agreement with our findings, as age was significantly associated with the prevalence of pulmonary fungal pathogens in PTB and non-PTB patients. We showed that the residential area was significantly associated with fungal infection in PTB and non-PTB individuals ($p < 0.05$). Participants from the rural areas had a relatively higher prevalence of fungal infections than those residing in the urban settlement. The occupations in rural areas might expose people much more to airborne fungal spores and yeast, reflecting the low socioeconomic status (SES) of the majority of participants in this study.

In our study, the prevalence of TB and fungal coinfection in males was higher (70.5%) compared to females (64.5%), which might be related to the more frequent engagement of men in outdoor activities [7, 31]. Also, the individuals who kept pets in their house showed a significantly higher risk of fungal infection ($p = 0.017$). The growing interest in keeping animals as pets in Nigeria highlights the possibility of zoonotic fungal transmission to humans [32]. Several reports have demonstrated domestic animals such as cats, dogs, sheep, horses, donkeys, and chickens as significant reservoirs of human fungal infections in Nigeria [33, 34]. Keeping pets is more common in people residing in rural and suburban settlements [32].

We found a higher pulmonary fungal infection rate (100%) among cigarette smokers ($n = 14$) compared to nonsmokers (71.1%) ($n = 140$), which is in agreement with a previous report of a significant association of smoking and fungal infections in the oral cavity in smokers [35]. Smoking is a predisposing factor in developing respiratory and oral bacterial and yeast infections [35, 36].

In this study, we had no chest image findings to confirm increased infiltrates or cavity formation in individuals with positive fungus culture. Also, we could not evaluate other comorbidities like chronic pulmonary disorders (COPD, bronchiectasis) and renal insufficiency

due to a lack of access to accurate diagnostic methods. Finally, this study was performed with a relatively small sample size, which might affect the inference from the statistical analysis.

We showed that PTB was a predisposing factor for secondary fungal infection, especially in low socioeconomic status persons. Government policymakers should implement socioeconomic measures to improve the health conditions of PTB patients. Individuals should also minimize risk factors of fungal infections reported in this study by quitting cigarette smoking, avoiding prolonged use of antibiotics, keeping healthy pets, and living in proper environmental hygiene.

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CONFLICT OF INTEREST

The authors declare that there is no conflict associated with this article.

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