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High Prevalence of Dihydropteroate Synthase Mutations in *Pneumocystis jirovecii* Isolated from Patients with *Pneumocystis* Pneumonia in South Africa▽

Leigh Dini,[1,2]* Mignon du Plessis,[3,4] John Frean,[4,5] and Victor Fernandez[1]

Department of Parasitology, Mycology, and Environmental Microbiology, Swedish Institute for Infectious Disease Control, Solna, Sweden[1]; School of Molecular and Cell Biology, University of the Witwatersrand, Johannesburg, South Africa[2]; Respiratory and Meningeal Pathogens Research Unit, National Institute for Communicable Diseases, Johannesburg, South Africa[3]; School of Pathology, University of the Witwatersrand, Johannesburg, South Africa[4]; and Parasitology Reference Unit, National Institute for Communicable Diseases, Johannesburg, South Africa[5]

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Pneumocystis jirovecii* pneumonia (PCP) is an important cause of morbidity and mortality in immunocompromised patients. Sulfa-containing drugs are used for the treatment and prophylaxis of PCP. Mutations in the *P. jirovecii fas* gene, which encodes dihydropteroate synthase (DHPS), are associated with prior exposure to sulfa drugs, and their appearance suggests the emergence of variants with reduced sulfa susceptibility. The present study examined the prevalence of DHPS mutations in *P. jirovecii* strains isolated from South African patients with PCP. *P. jirovecii* infection was investigated by immunofluorescence microscopy and quantitative real-time PCR with respiratory specimens from 712 patients (93% of whom were >15 years of age) with suspected PCP consecutively received for the detection of *P. jirovecii* over 1 year. PCR amplification and sequencing of the DHPS *fas* gene was attempted with DNA from the *P. jirovecii*-positive samples. *P. jirovecii* infection was confirmed by immunofluorescence microscopy in 168/712 (24%) of the patients. Carriage of the fungus was revealed by real-time PCR in 17% of the patients with negative microscopy results. The *P. jirovecii fas* gene was successfully amplified from specimens from 151 patients and sequenced. Mutations resulting in the Thr55Ala and/or Pro57Ser amino acid substitution were detected in *P. jirovecii* strains from 85/151 (56%) patients. The high frequency of PCP episodes with *P. jirovecii* harboring DHPS mutations in South Africa indicates that populations of this fungus are evolving under the considerable selective pressure exerted by sulfa-containing antibiotics. These results, similar to previous observations of sulfa drug resistance in bacterial populations, underscore the importance of the rational use of sulfa medications either prophylactically against PCP or for the treatment of other infections.

Pneumocystis pneumonia (PCP), a major opportunistic infection in immunocompromised patients, is caused by the fungus *Pneumocystis jirovecii*. The incidence of PCP, which increased dramatically with the advent of the HIV/AIDS pandemic, has decreased in the industrialized world owing to the widespread use of sulfa drug prophylaxis and the introduction of highly active antiretroviral therapy (HAART). However, PCP remains an important cause of morbidity and mortality in HIV/AIDS patients, as well as in immunocompromised non-HIV-infected patients, in whom its incidence is increasing (17, 35). In South Africa, which has a population of 48.5 million, an estimated 5.7 million people were living with HIV in 2007, and 350,000 deaths were attributed to AIDS during the same year (20). The South African government initiated the provision of HAART to the public sector in April 2004, but prior to that, the HIV epidemic was largely untreated. By the end of 2006, the rate of HAART coverage was estimated to be 21% among those needing antiretroviral therapy (20). Studies from African countries report variable incidences of PCP in adult patients with HIV/AIDS and generally higher rates in children (1, 3, 27, 28, 43, 47, 49). In South Africa, where a limited number of laboratories offer testing for *P. jirovecii*, the vast majority of PCP cases are diagnosed clinically and radiologically.

Sulfonamides, usually combined with trimethoprim, as in trimethoprim-sulfamethoxazole (TMP-SMX), and dapsone are used for the treatment and prophylaxis of PCP. There are few alternative drugs for the treatment of this infection. Sulfonamides inhibit the enzyme dihydropteroate synthase (DHPS), an essential component of the folate synthesis pathway (36). In *P. jirovecii*, two nonsynonymous point mutations in the *fas* gene, which encodes the DHPS enzyme, are associated with prior exposure to sulfa drugs (5, 15, 16, 22, 26, 32), and concerns have been raised about the possible emergence of resistance to sulfa drugs (38). These mutations, at nucleotide positions 165 and 171, cause the amino acid substitutions Thr55Ala and Pro57Ser in the DHPS protein, respectively. Point mutations in the DHPS-encoding genes of microorganisms such as *Plasmodium falciparum*, *Staphylococcus aureus*, *Mycobacterium leprae*, and *Escherichia coli* have been shown to confer resistance to sulfonamides (6, 14, 21, 45). As *P. jirovecii* cannot yet be cultured, conventional *in vitro* susceptibility tests cannot be utilized; therefore, studies of drug resistance in this organism rely on the use of genetic markers and suitable models. Functional complementation of either DHPS-disrupted *E.*

* Corresponding author. Mailing address: Swedish Institute for Infectious Disease Control, Nobelsväg 18, Solna 17182, Sweden. Phone: 46-8-4572498. Fax: 46-8-318450. E-mail: leigh.dini@smi.se.

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coli with a mutant *P. jirovecii fas* gene or FOL1-disrupted *Saccharomyces cerevisiae* with the *fol1* gene mutated at positions analogous to positions 165 and 171 in *fas* results in the loss of susceptibility to sulfamethoxazole and other sulfa-containing drugs (19, 29).

The prevalence of *P. jirovecii* DHPS mutations reported from countries in the developed world ranges widely, from 4% to 81% (2, 5, 15, 18, 32, 39, 41, 42). In South Africa, a few studies that screened specimens from limited numbers of adults and children have reported mostly low mutation rates (8, 12, 34, 48). Here we present the results of a large laboratory-based study aimed at ascertaining the prevalence in South Africa of *P. jirovecii* strains harboring mutations at positions 165 and 171 in the *fas* gene.

MATERIALS AND METHODS

Laboratory setting. The Parasitology Reference Unit of the National Institute for Communicable Diseases (NICD), in Johannesburg, South Africa, is the largest recipient of referrals for the microbiological confirmation of *P. jirovecii* infection in the country, receiving about 1,000 specimens annually.

Clinical specimens and patient information. Respiratory specimens ($n = 932$) from patients with suspected PCP ($n = 712$) were referred to the NICD from 38 public hospitals and clinics in six provinces of South Africa from January 2006 through February 2007. Data regarding patient age, gender, and hospital location were retrospectively extracted from a laboratory information system. The mean age of the patients was 34 ± 13 years, with the age range being 1 month to 86 years (the ages of 81 patients were unknown). The ratio of females to males was 1.6:1 (the genders of 18 patients were unknown). The majority of patients ($n = 491/712$, 69%) were from Gauteng Province; and the rest were from the Eastern Cape, Mpumalanga, Free State, Limpopo, and Northern Cape Provinces. The majority of specimens received for the detection of *P. jirovecii* were sputa ($n = 839/932$, 90%). When multiple specimens from the same patient and PCP episode were provided, only the first *P. jirovecii*-positive specimen received in the laboratory was included in the analysis. If more than one specimen was taken on the same day, the specimen with the higher fungal load was included in the analysis. Data concerning HIV status and prophylaxis and/or treatment for PCP were not available for these patients. All procedures followed were in accordance with the ethical standards of the Human Research Ethics Committee (Medical) of the University of the Witwatersrand in Johannesburg (protocol no. M050320).

Specimen processing and laboratory detection of *P. jirovecii*. Sputum specimens and other specimen types containing mucus were pretreated with 0.1% dithiothreitol (Sigma-Aldrich, South Africa). After centrifugation, microscopic slides were prepared with the sediment. The remaining sediment was washed with phosphate-buffered saline and stored at −70°C prior to DNA extraction. The slides were stained with a direct fluorescent antibody (for immunofluorescence [IF] microscopy) specific for the detection of *P. jirovecii* cysts (Light Diagnostics, Millipore, Temecula, CA), according to the manufacturer's instructions. A positive IF microscopy result was defined as the presence in a specimen of at least one cluster of *P. jirovecii* cysts with the characteristic morphology and fluorescent immunostaining pattern. An equivocal result was defined as the presence in a specimen of a cyst that had a suspicious *Pneumocystis*-like cyst morphology but that did not meet the criteria for confirmation of a positive result.

DNA extraction. DNA was extracted from 50 μl of sediment, which was digested with proteinase K at 56°C for 2 h, prior to automated DNA extraction with an M48 BioRobot apparatus (Qiagen, Germany), according to the manufacturer's protocol for soft tissue. The purified DNA was eluted in 100 μl of sterile water and stored at −20°C.

Determination of *P. jirovecii* DNA load. The amount of *P. jirovecii* DNA in specimens was determined by a quantitative real-time PCR (qPCR) targeting the mitochondrial gene coding for the large ribosomal subunit (mtLSU) of the fungus. The following primers and probe were used: LSU1 (5′-AAA TAA ATA ATC AGA CTA TGT GCG ATA AGG-3′), LSU2 (5′-GGG AGC TTT AAT TAC TGT TCT GGG-3′), and LSUP1 (6-carboxyfluorescein–5′-AGA TAG TCC AAA GGG AAA C-3′–6-carboxytetramethylrhodamine) (Applied Biosystems, Foster City, CA). The amplification reactions were carried out in a total volume of 25 μl containing 1× TaqMan universal PCR master mixture, 0.25 μl of a mixture of primers and probe at 20 μM each, 5 μl of template DNA, and RNase-free water to complete the volume. Thermal cycling was performed in an Applied Biosystems GeneAmp PCR system 7900 thermocycler, as follows: 2 min at 50°C and 10 min at 95°C, followed by 45 cycles of 15 s at 95°C and 1 min at 60°C. Quantification was based on extrapolation from standard curves generated by amplification of serial dilutions of linearized plasmid pCR2.1 containing the *P. jirovecii* DNA fragment defined by primers LSU1 and LSU2.

Analysis of DHPS genotypes. Specimens with ≥20 target mtLSU gene copies/μl of extracted DNA were selected for DHPS analysis, as the amplification of the single-copy DHPS *fas* gene proved to be less sensitive than the real-time PCR assay. Primers SMIF1 (5′-CAA ATT AGC GTA TCG AAT GAC C-3′) and SMIB2 (5′-GCA AAA TTA CAA TCA ACC AAA GTA-3′) were used to amplify a 1,030-bp region of the DHPS gene. Subsequently, nested primers SMIF6 (5′-AGC GCC TAC ACA TAT TAT GG-3′) and SMIB7 (5′-GTT CTG CAA CCT CAG AAC G-3′) were used to amplify a 278-bp region encompassing polymorphic nucleotide positions ^{165}A/G and ^{171}C/T. PCR amplification was performed in a Mastercycler gradient apparatus (Eppendorf AG, Hamburg, Germany) by using the following conditions for the primary PCR: 94°C for 5 min, followed by 35 cycles at 94°C for 30 s, 55°C for 1 min, and 72°C for 2 min and a final extension at 72°C for 7 min. The nested PCR conditions were 94°C for 5 min, followed by 35 cycles at 94°C for 30 s, 52°C for 30 s, and 72°C for 1 min and a final extension at 72°C for 7 min. The PCR products were visualized by gel electrophoresis on 2% agarose gels. The nested PCR products were sequenced by using primer SMIF6 and BigDye Terminator chemistry.

Measures taken to prevent contamination of DNA. Different laboratories were used for the initial processing and immunostaining of the specimens, DNA extraction, addition of the PCR product to the nested reaction mixture, and gel electrophoresis. A laboratory free of *Pneumocystis* DNA was used to prepare all PCR master mixtures. The DNA was handled in a laminar flow cabinet equipped with a UV lamp for decontamination. Positive and negative controls were included with the DNA extraction process and PCRs.

Data analysis. The DNA sequences were analyzed with BioEdit sequence alignment editor software (version 7.0.9.0). Statistical analyses were performed with the Excel program. The chi-square test was used to assess significant ($P < 0.05$) associations among categorical variables.

RESULTS

During the study period, from January 2006 through February 2007, 932 respiratory specimens from 712 patients (93% of whom were >15 years of age) with suspected PCP were consecutively received for the microbiological confirmation of PCP at the NICD in Johannesburg, South Africa. IF microscopy was positive for *P. jirovecii* in specimens from 168 (24%) patients. *P. jirovecii* DNA was detected in all of the IF microscopy-positive specimens from 150 patients that were available for assessment of the fungal load by qPCR. The quantity of *P. jirovecii* DNA measured in the first IF microscopy-positive specimen from each of these 150 patients is shown in Table 1. The IF microscopy result was deemed equivocal for specimens from 14 patients, and 10 of these yielded positive results by qPCR (Table 1). Specimens from 530 patients were IF microscopy negative for *P. jirovecii*. Quantitative PCR was performed with a subset of IF microscopy-negative specimens from 191 patients that were arbitrarily chosen throughout the year. *P. jirovecii* DNA was detected in IF microscopy-negative specimens from 32 (17%) of these patients and quantified (Table 1).

Amplification of the DHPS gene of *P. jirovecii* was attempted in 201 specimens from 162 patients with a qPCR result of ≥20 target gene copies/μl of extracted DNA. The DHPS locus was successfully genotyped in a total of 183 specimens from 151 patients. Point mutations resulting in the Thr55Ala and/or Pro57Ser amino acid substitution were detected in *P. jirovecii* isolates from 85 (56%) patients (Table 2). Of these, 36 patients (42%) harbored *P. jirovecii* isolates with either the Thr55Ala mutation (19 patients, 22%) or the Pro57Ser mutation (17 patients, 20%). In specimens from 18

TABLE 1. Quantification of *P. jirovecii* DNA in respiratory specimens from South African patients with suspected *Pneumocystis* pneumonia

Specimen and IF microscopy result	No. of patients	No. of patients qPCR positive	Median (range) *P. jirovecii* DNA quantity[a]
Sputum			
Positive	140	140	3,062 (8–291,894)
Negative[b]	173	30	7 (1–499)
Equivocal[c]	12	10	151 (9–1,549)
Tracheal aspirate			
Positive	7	7	1,921 (10–180,498)
Negative	4	1	0.8
Equivocal	1	0	
Bronchoalveolar lavage			
Positive	2	2	54,766 (3–109,529)
Negative	3	0	
Other[d]			
Positive	1	1	299
Negative	11	1	4
Equivocal	1	0	

[a] Expressed as the target number of gene copies/μl of extracted DNA. Detailed information regarding the handling of specimens and DNA extraction conditions is provided in Materials and Methods.
[b] A set of 205 specimens from 191 patients from among the 530 patients with IF microscopy-negative results was arbitrarily chosen for qPCR analysis.
[c] Immunofluorescence microscopy was suggestive of *P. jirovecii*, but the result was not conclusive.
[d] Includes two bronchial washings, two nasopharyngeal aspirates, one pleural fluid specimen, and eight samples of unknown specimen types.

TABLE 2. *Pneumocystis jirovecii* DHPS genotypes in respiratory specimens from South African patients

DHPS genotype	Nucleotide at position (amino acid):		No. of patients positive/total no. of patients (%) (n = 151)
	165 (55)	171 (57)	
Wild type	A (Thr)	C (Pro)	66/151 (44)
Mutant genotype, total			85/151 (56)
Single mutation			36/85 (42)
Position 165 (M1)	G (Ala)	C (Pro)	4/85 (5)
M1 + wild type	G/A (Ala/Thr)	C (Pro)	15/85 (18)
Position 171 (M2)	A (Thr)	T (Ser)	7/85 (8)
M2 + wild type	A (Thr)	T/C (Ser/Pro)	10/85 (12)
Double mutation			18/85 (21)
Positions 165 + 171 (M3)	G (Ala)	T (Ser)	15/85 (18)
M3 + M1	G (Ala)	T/C (Ser/Pro)	1/85 (1)
M3 + M2	G/A (Ala/Thr)	T (Ser)	2/85 (2)
Mixed genotypes[a]	G/A (Ala/Thr)	T/C (Ser/Pro)	31/85 (36)

[a] Mixed infections in which the presence of mutants with double mutations and the wild type and/or a mixture of mutants with single mutations could not be resolved.

patients (21%), the presence of *P. jirovecii* organisms with both mutations in the DHPS gene could be ascertained. Specimens from 31 patients (36%) contained mixtures of mutant *P. jirovecii* DHPS genotypes, both with and without the wild-type genotype, which could not be resolved by direct sequencing. *P. jirovecii* infections with more than one DHPS genotype were detected in 69% (59/85) of patients harboring mutant *P. jirovecii* strains. Multiple respiratory specimens collected during the same PCP episode were available from 23 patients, and strains in the majority of the specimens (17/23, 74%) presented identical DHPS genotypes. There were no significant differences in the frequency of wild-type or mutant DHPS genotypes between genders ($P = 0.39$), age groups ($P = 0.38$), or patients from distinct geographical locations ($P = 0.1$).

DISCUSSION

The primary aim of the present study was to assess the prevalence in South Africa of *P. jirovecii* strains harboring DHPS mutations previously shown to be associated with exposure to sulfa or sulfone drugs. The number of specimens and patients screened makes this study the largest of its kind conducted in the developing world to date. We found that 56% of patients with PCP were infected with mutant *P. jirovecii* strains. This proportion stands in contrast to the lower frequencies reported from the African continent and other countries outside the industrialized world (8, 12, 23, 25, 30, 34, 40, 46, 48). The risk that *P. jirovecii* will develop mutations in the DHPS gene is higher for patients with PCP receiving sulfa drug prophylaxis than for those not receiving it (5, 15, 16, 22, 26, 32, 38). A general assumption is that the frequency of occurrence of mutations in the DHPS gene in developing countries is low due to the less extensive use of sulfa drug prophylaxis against PCP. Thus, the high frequency of mutations in the DHPS gene among strains from PCP patients in South Africa would be indicative of a considerable exposure to sulfa medication used either prophylactically against PCP or for the treatment of other infections. We regard it as unlikely that sulfa drug (usually TMP-SMX) prophylaxis for PCP alone accounts for the high prevalence of mutant *P. jirovecii* strains observed in the present investigation. Although information regarding the HIV infection status of the patients and the use of prophylaxis was not available for this laboratory-based study, it is well recognized that a large proportion of HIV-positive patients in South Africa are either unaware of their HIV infection when they present with PCP or have limited access to prophylaxis. During a large clinical study in progress at a public hospital in Johannesburg, interim results showed that 58% of adult patients presenting with PCP were newly diagnosed with HIV infection and that only 27% of patients with a previously confirmed HIV infection were receiving TMP-SMX prophylaxis (M. Wong, personal communication). Furthermore, in a survey of South African primary-level health care services for HIV-positive children, two-thirds of the public-sector clinics did not have in place a policy for the administration of PCP prophylaxis (11).

The widespread use of sulfa drugs, particularly TMP-SMX, against diseases other than PCP could be contributing to the selection of *P. jirovecii* strains carrying mutations in the DHPS gene. In South Africa, guidelines issued by the Department of Health have recommended the use of TMP-SMX to treat urinary tract infections, cholera, and acute otitis media at the

primary-care level, as well as invasive bacterial infections caused by *Shigella* and *Salmonella* species in hospitalized patients (37). Indications for HIV-positive individuals, apart from PCP prophylaxis and treatment, include prophylaxis against recurrent bacterial infections in children; prophylaxis against bacterial pneumonia, bacteremia, and isosporiasis in adults; as well as the prophylaxis and treatment of toxoplasmosis. A 2002 report revealed that 26% of primary health care clinics also administered TMP-SMX for the treatment of acute respiratory and gastrointestinal tract infections in children (11), a finding in line with the generalized perception that sulfa-based drugs are often used in primary care as empirical treatment for a variety of conditions, including diarrhea and nonspecific respiratory infections in adults and children. The extensive usage of TMP-SMX is known to exert considerable selective pressure on microbial populations and lead to the development of resistance (4, 9). That this is the situation in Gauteng Province and other localities of South Africa is also suggested by the very high rates of TMP-SMX resistance consistently observed in surveillance isolates of pneumococcal and other bacterial species; for example, rates of TMP-SMX resistance in isolates of *Streptococcus pneumoniae*, *Shigella* spp., and nontyphoidal salmonellae of over 50%, 83%, and 37%, respectively, have been reported (13, 44). Sulfadoxine-pyrimethamine (Fansidar) was used as the first-line treatment for malaria in South Africa until 2006; while the number of patients treated (between 1990 and 2006, an average of about 25,000 malaria cases per year) was relatively small in comparison to the burden of HIV-infected individuals, this may have contributed to selective pressure.

The transmission of *P. jirovecii* strains is another factor that may influence the high prevalence of mutant DHPS strains observed in the present investigation. The airborne transmission of *Pneumocystis* spp. from host to host has been demonstrated in rodent models; and numerous observations, including several cases of nosocomial clustering, suggest that direct or indirect transmission occurs in humans (33). Evidence for the *de novo* acquisition of *P. jirovecii* with mutant DHPS genotypes has been provided by studies that included patients who had never received sulfa drug prophylaxis for PCP (16, 24). In one of those studies, more than half of the patients newly diagnosed with HIV infection harbored mutant *P. jirovecii* strains (16). It is possible that the interhuman transmission of *P. jirovecii* and the concomitant circulation of DHPS genotypes in the population are accelerated in localities of South Africa with a high prevalence of HIV infection and large numbers of individuals with some degree of immunosuppression, from subclinical stages to overt AIDS, who are susceptible to colonization by the fungus. The widespread empirical use of TMP-SMX and the rates of increased transmission and circulation of strains/genotypes in the population could explain, at least in part, the high prevalence of mutant *P. jirovecii* strains in settings in which the implementation of PCP prophylaxis is limited.

A lack of clinical surveillance, the lack of availability of chest radiographic facilities, and limited laboratory diagnostic capabilities contribute to the scarcity and disparity of information regarding the burden of disease and the epidemiology of PCP in African settings. In South Africa, the collection of bronchoalveolar lavage (BAL) specimens is rare. The few specimens sent for laboratory confirmation are mostly sputa, and these are often spontaneously expectorated rather than induced with hypertonic saline (7, 10). Although the collection procedure was not specified for the majority of sputum specimen referrals in this study, we estimate that a high proportion of specimens were spontaneously expectorated. Clinically, it is accepted that BAL fluid and induced sputum specimens are superior to spontaneously expectorated sputum for the microscopic diagnosis of PCP, in part because many patients with PCP have a nonproductive cough and cannot produce an adequate specimen without undergoing sputum induction. While the proper collection of the respiratory sample is of major importance for the optimal laboratory diagnosis of PCP, it may also be crucial for the strain/genotype representativity of the specimen and the accurate assessment of mixed-genotype infections. In five of a total of six cases with discrepant DHPS genotypes, we observed large differences ($\geq$10-fold) in the quantity of *P. jirovecii* DNA measured in specimens from the same PCP episode caused by strains showing nonidentical DHPS genotypes. In some of these cases, multiple samples had been collected within 24 h or less. This suggests that variable specimen quality, as well as initiation of PCP therapy, may be a cause of inconsistent results in the genotypic analysis of *P. jirovecii* infections.

In South Africa, PCP has been studied more extensively in children, and *P. jirovecii* is now recognized as a common pathogen in HIV-infected infants (49). In the present study, which predominantly included specimens from adults, *P. jirovecii* infection was confirmed by IF microscopy in 24% of patients with suspected PCP. By quantitative real-time PCR analysis, we could confirm *P. jirovecii* carriage in an additional 17% of patients with a clinical suspicion of PCP and negative IF microscopy results. The detection of *P. jirovecii* was also verified by real-time PCR in specimens from 10 of 14 patients with equivocal microscopy results. Quantitative real-time PCR performed in a closed system offers the advantages of increased diagnostic sensitivity and rapidity with a reduced risk of contamination. Quantification of the level of *P. jirovecii* infection or colonization can be useful for the monitoring of therapy and the improved management of patients (31). Although increased diagnostic sensitivity carries a higher risk of clinically false-positive results, it enhances the chances for the early detection of biologically true-positive results for individuals with no or incipient clinical manifestations, something that could be of critical importance in localities with a high incidence of HIV infection.

This study has certain limitations. Data regarding HIV infection status, the use of sulfa drug prophylaxis or some other medication, and the outcomes for the patients were not available. These shortcomings, which preclude further analysis of possible underlying associations, expose the types of constraints that are common for laboratory-based studies. However, they do not have any bearing on the central issue of assessing the frequency of DHPS genotypes in *P. jirovecii* strains prevalent in the population. Although the study included patients from a large number of hospitals in South Africa, 57% of the patients for whom data on *P. jirovecii* DHPS genotypes were available came from two hospitals in Gauteng Province.

The increased risk that patients receiving sulfa drug prophy-

laxis carry mutant *P. jirovecii* DHPS genotypes suggests that mutations in the *fas* gene provide a selective advantage so that subpopulations of the fungus may eventually overcome the effects of prophylactic doses of TMP-SMX. The clinical significance of DHPS mutations and, in particular, whether single polymorphisms or double mutations have differential effects on the response to higher therapeutic doses are questions not yet resolved. The appearance of *P. jirovecii* strains with additional mutations and significantly higher levels of tolerance to sulfonamides and/or sulfones is a possibility that cannot be excluded. Examples of cumulative mutations leading to increased antibiotic resistance have been documented for viruses, bacteria, and parasitic protozoa.

In summary, the results of the present investigation show that the frequency of infections with *P. jirovecii* strains harboring DHPS mutations is high in adult patients in South Africa. This finding suggests the existence of considerable antimicrobial pressure favoring the establishment in the population of *P. jirovecii* strains with reduced sensitivities to sulfa drugs. The most likely source of this selective pressure is the utilization of TMP-SMX, both prophylactically against PCP and for the treatment of other infections. The importance of regular monitoring for DHPS gene polymorphisms and the rational use of sulfa medications is underscored.

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REFERENCES

1. **Aderaye, G., J. Bruchfeld, G. Aseffa, Y. Nigussie, K. Melaku, Y. Woldeamanuel, D. Asrat, A. Worku, H. Gaegziabher, M. Lebaad, and L. Lindquist.** 2007. *Pneumocystis jirovecii* pneumonia and other pulmonary infections in TB smear-negative HIV-positive patients with atypical chest X-ray in Ethiopia. Scand. J. Infect. Dis. **39:**1045–1053.
2. **Alvarez-Martinez, M. J., A. Moreno, J. M. Miro, M. E. Valls, P. V. Rivas, E. de Lazzari, O. Sued, N. Benito, P. Domingo, E. Ribera, M. Santin, G. Sirera, F. Segura, F. Vidal, F. Rodriguez, M. Riera, M. E. Cordero, J. R. Arribas, M. T. J. de Anta, J. M. Gatell, P. E. Wilson, S. R. Meshnick, and Spanish PCP Working Group.** 2008. *Pneumocystis jirovecii* pneumonia in Spanish HIV-infected patients in the combined antiretroviral therapy era: prevalence of dihydropteroate synthase mutations and prognostic factors of mortality. Diagn. Microbiol. Infect. Dis. **62:**34–43.
3. **Chakaya, J. M., C. Bii, L. Ng'ang'a, E. Amukoye, T. Ouko, L. Muita, S. Gathua, J. Gitau, I. Odongo, J. M. Kabanga, K. Nagai, S. Suzumura, and Y. Sugiura.** 2003. *Pneumocystis carinii* pneumonia in HIV/AIDS patients at an urban district hospital in Kenya. East Afr. Med. J. **80:**30–35.
4. **Chenoweth, C. E., S. Saint, F. Martinez, J. P. Lynch III, and A. M. Fendrick.** 2000. Antimicrobial resistance in *Streptococcus pneumoniae*: implications for patients with community-acquired pneumonia. Mayo Clin. Proc. **75:**1161–1168.
5. **Crothers, K., C. B. Beard, J. Turner, G. Groner, M. Fox, A. Morris, S. Eiser, and L. Huang.** 2005. Severity and outcome of HIV-associated *Pneumocystis* pneumonia containing *Pneumocystis jirovecii* dihydropteroate synthase gene mutations. AIDS **19:**801–805.
6. **Dallas, W. S., J. E. Gowen, P. H. Ray, M. J. Cox, and I. K. Dev.** 1992. Cloning, sequencing, and enhanced expression of the dihydropteroate synthase gene of *Escherichia coli* MC4100. J. Bacteriol. **174:**5961–5967.
7. **Dini, L., and J. Frean.** 2000. Diagnosis and management of *Pneumocystis carinii* pneumonia. S. Afr. J. Epidemiol. Infect. **15:**23–26.
8. **Dini, L., M. L. Wong, M. du Plessis, A. Karstaedt, V. Fernandez, and J. Frean.** 2006. Prevalence of DHPS polymorphisms associated with sulfa resistance in South African *Pneumocystis jirovecii* strains. J. Eukaryot. Microbiol. **53**(Suppl. 1)**:**S110–S111.
9. **Felmingham, D.** 2004. Comparative antimicrobial susceptibility of respiratory tract pathogens. Chemotherapy **50**(Suppl. 1)**:**3–10.
10. **Frean, J. A., and L. A. Dini.** 2000. *Pneumocystis carinii* in South Africa. S. Afr. Med. J. **90:**828–829.
11. **Giese, S., and G. Hussey.** 2002. Rapid appraisal of primary level health care services for HIV-positive children at public sector clinics in South Africa. Research report. Children's Institute, University of Cape Town, Cape Town, South Africa.
12. **Govender, S., S. J. du Plessis, G. S. Ocana, and L. J. Chalkley.** 2008. Prevalence of *Pneumocystis jirovecii* and *Mycoplasma pneumoniae* in patients presenting with pneumonia at hospitals in Port Elizabeth. S. Afr. J. Epidemiol. Infect. **23:**21–24.
13. **Group for Enteric, Respiratory and Meningeal Disease Surveillance in South Africa.** 2008. GERMS-SA annual report 2007. http://www.nicd.ac.za/units/germs/germs.htm. Accessed 30 June 2009.
14. **Hampele, I. C., A. D'Arcy, G. E. Dale, D. Kostrewa, J. Nielsen, C. Oefner, M. G. Page, H. J. Schönfeld, D. Stüber, and R. L. Then.** 1997. Structure and function of the dihydropteroate synthase from *Staphylococcus aureus*. J. Mol. Biol. **268:**21–30.
15. **Helweg-Larsen, J., T. L. Benfield, J. Eugen-Olsen, J. D. Lundgren, and B. Lundgren.** 1999. Effects of mutations in *Pneumocystis carinii* dihydropteroate synthase gene on outcome of AIDS-associated *P. carinii* pneumonia. Lancet **354:**1347–1351.
16. **Huang, L., C. B. Beard, J. Creasman, D. Levy, J. S. Duchin, S. Lee, N. Pieniazek, J. L. Carter, C. del Rio, D. Rimland, and T. R. Navin.** 2000. Sulfa or sulfone prophylaxis and geographic region predict mutations in the *Pneumocystis carinii* dihydropteroate synthase gene. J. Infect. Dis. **182:**1192–1198.
17. **Huang, L., L. Morris, A. H. Limper, J. M. Beck, and ATS *Pneumocystis* workshop participants.** 2006. An official ATS workshop summary: recent advances and future directions in *Pneumocystis* pneumonia (PCP). Proc. Am. Thorac. Soc. **3:**655–664.
18. **Huang, L., D. A. Welsh, R. F. Miller, C. B. Beard, G. G. Lawrence, M. Fox, A. Swartzman, M. R. Bensley, D. Carbonnet, J. L. Davis, A. Chi, B. J. Yoo, and J. L. Jones.** 2006. *Pneumocystis jirovecii* dihydropteroate synthase gene mutations and human immunodeficiency virus-associated *Pneumocystis* pneumonia. J. Eukaryot. Microbiol. **53**(Suppl. 1)**:**S114–S116.
19. **Iliades, P., S. R. Meshnick, and I. G. Macreadie.** 2005. Mutations in the *Pneumocystis jirovecii* DHPS gene confer cross-resistance to sulfa drugs. Antimicrob. Agents Chemother. **49:**741–748.
20. **Joint United Nations Programme on HIV/AIDS and the World Health Organization.** 2008. Epidemiological fact sheet on HIV and AIDS, South Africa, 2008 update. http://www.who.int/hiv. Accessed 1 December 2008.
21. **Kai, M., M. Matsuoka, N. Nakata, S. Maeda, M. Gidoh, Y. Maeda, K. Hashimoto, K. Kobayashi, and Y. Kashiwabara.** 1999. Diaminodiphenylsulfone resistance of *Mycobacterium leprae* due to mutations in the dihydropteroate synthase gene. FEMS Microbiol. Lett. **177:**231–235.
22. **Kazanjian, P., W. Armstrong, P. A. Hossler, W. Burman, J. Richardson, C. H. Lee, L. Crane, J. Katz, and S. R. Meshnick.** 2000. *Pneumocystis carinii* mutations are associated with duration of sulfa or sulfone prophylaxis exposure in AIDS patients. J. Infect. Dis. **182:**551–557.
23. **Kazanjian, P. H., D. Fisk, W. Armstrong, Q. Shulin, H. Liwei, Z. Ke, and S. Meshnick.** 2004. Increase in prevalence of *Pneumocystis carinii* mutations in patients with AIDS and *P. carinii* pneumonia, in the United States and China. J. Infect. Dis. **189:**1684–1687.
24. **Latouche, S., P. Lacube, E. Maury, J. Bolognini, M. Develoux, P. Girard, C. Godet, M. Lebrette, C. Mayaud, J. Guillot, and P. Roux.** 2003. *Pneumocystis jirovecii* dihydropteroate synthase genotypes in French patients with pneumocystosis: a 1998–2001 prospective study. Med. Mycol. **41:**533–537.
25. **Li, K., A. He, W. P. Cai, X. P. Tang, X. Y. Zheng, Z. Y. Li, and X. M. Zhan.** 2009. Absence of *Pneumocystis jirovecii* dihydropteroate synthase gene mutations among samples from a group of AIDS patients in China. Scand. J. Infect. Dis. **41:**152–154.
26. **Ma, L., J. A. Kovacs, A. Cargnel, A. Valerio, G. Fantoni, and C. Atzori.** 2002. Mutations in the dihydropteroate synthase gene of human-derived *Pneumocystis carinii* isolates from Italy are infrequent but correlate with prior sulfa prophylaxis. J. Infect. Dis. **185:**1530–1532.
27. **Mahomed, A. G., J. Murray, S. Klempman, G. Richards, C. Feldman, N. T. Levy, C. Smith, and J. Kallenbach.** 1999. *Pneumocystis carinii* pneumonia in HIV infected patients from South Africa. East Afr. Med. J. **76:**80–84.
28. **Malin, A. S., L. K. Z. Gwanzura, S. Klein, V. J. Robertson, P. Musvaire, and P. R. Mason.** 1995. *Pneumocystis carinii* pneumonia in Zimbabwe. Lancet **346:**1258–1261.
29. **Meneau, I., D. Sanglard, J. Bille, and P. M. Hauser.** 2004. *Pneumocystis jirovecii* dihydropteroate synthase polymorphisms confer resistance to sulfadoxine and sulfanilamide in *Saccharomyces cerevisiae*. Antimicrob. Agents Chemother. **48:**2610–2616.
30. **Miller, R. F., A. R. Lindley, H. E. Ambrose, A. S. Malin, and A. E. Wakefield.** 2003. Genotypes of *Pneumocystis jirovecii* isolates obtained in Harare, Zimbabwe, and London, United Kingdom. Antimicrob. Agents Chemother. **47:**3979–3981.
31. **Mori, S., I. Cho, and M. Sugimoto.** 2009. A followup study of asymptomatic

carriers of *Pneumocystis jiroveci* during immunosuppressive therapy for rheumatoid arthritis. J. Rheumatol. **36:**1600–1605.
32. **Nahimana, A., M. Rabodonirina, G. Zanetti, I. Meneau, P. Francioli, J. Bille, and P. M. Hauser.** 2003. Association between a specific *Pneumocystis jiroveci* dihydropteroate synthase mutation and failure of pyrimethamine/sulfadoxine prophylaxis in human immunodeficiency virus-positive and -negative patients. J. Infect. Dis. **188:**1017–1023.
33. **Nevez, G., M. Chabé, M. Rabodonirina, M. Virmaux, E. Dei-Cas, P. M. Hauser, and A. Totet.** 2008. Nosocomial *Pneumocystis jirovecii* infections. Parasite **15:**359–365.
34. **Robberts, F. J., L. J. Chalkley, K. Weyer, P. Goussard, and L. D. Liebowitz.** 2005. Dihydropteroate synthase and novel dihydrofolate reductase gene mutations in strains of *Pneumocystis jirovecii* from South Africa. J. Clin. Microbiol. **43:**1443–1444.
35. **Santamauro, J. T., R. N. Aurora, and D. E. Stover.** 2002. *Pneumocystis carinii* pneumonia in patients with and without HIV infection. Comp. Ther. **28:**96–108.
36. **Sköld, O.** 2000. Sulfonamide resistance: mechanisms and trends. Drug Resist. Updat. **3:**155–160.
37. **South Africa Department of Health.** 2008, posting date. Standard treatment guidelines and essential drugs list for South Africa. http://www.doh.gov.za/docs/. Accessed 11 May 2009.
38. **Stein, C. R., C. Poole, P. Kazanjian, and S. R. Meshnick.** 2004. Sulfa use, dihydropteroate synthase mutations, and *Pneumocystis jirovecii* pneumonia. Emerg. Infect. Dis. **10:**1760–1765.
39. **Takahashi, T., N. Hosoya, T. Endo, T. Nakamura, H. Sakashita, K. Kimura, K. Ohnishi, Y. Nakamura, and A. Iwamoto.** 2000. Relationship between mutations in dihydropteroate synthase of *Pneumocystis carinii* f. sp. *hominis* isolates in Japan and resistance to sulfonamide therapy. J. Clin. Microbiol. **38:**3161–3164.
40. **Tyagi, A. K., B. R. Mirdha, R. Guleria, A. Mohan, K. Luthra, and U. B. Singh.** 2008. Study of dihydropteroate synthase (DHPS) gene mutations among isolates of *Pneumocystis jiroveci*. Indian J. Med. Res. **128:**734–739.
41. **Valerio, A., E. Tronconi, F. Mazza, G. Fantoni, C. Atzori, F. Tartarone, P. Duca, and A. Cargnel.** 2007. Genotyping of *Pneumocystis jirovecii* pneumonia in Italian AIDS patients. J. Acquir. Immune Defic. Syndr. **45:**521–528.
42. **Van Hal, S. J., F. Gilgado, T. Doyle, J. Barratt, D. Stark, W. Meyer, and J. Harkness.** 2009. Clinical significance and phylogenetic relationship of novel Australian *Pneumocystis jirovecii* genotypes. J. Clin. Microbiol. **47:**1818–1823.
43. **Van Oosterhout, J. J. G., M. K. Laufer, M. A. Perez, S. M. Graham, N. Chimbiya, P. C. Thesing, M. J. Alvarez-Martinez, P. E. Wilson, M. Chagomerana, E. E. Zijlstra, T. E. Taylor, C. V. Plowe, and S. R. Meshnick.** 2007. *Pneumocystis* pneumonia in HIV-positive adults, Malawi. Emerg. Infect. Dis. **13:**325–328.
44. **von Gottberg, A.** 3 March 2009, posting date. Laboratory-based surveillance for invasive pneumococcal disease in South Africa. http://www.sabin.org/files/Anne%20von%20Gottberg%204pneumo%20revised.pdf. Accessed 14 July 2009.
45. **Wang, P., M. Read, P. F. Sims, and J. E. Hyde.** 1997. Sulfadoxine resistance in the human parasite *Plasmodium falciparum* is determined by mutations in dihydropteroate synthetase and an additional factor associated with folate utilisation. Mol. Microbiol. **23:**979–986.
46. **Wissmann, G., M. J. Alvarez-Martinez, S. R. Meshnick, A. R. S. Dihel, and J. C. Prolla.** 2006. Absence of dihydropteroate synthase mutations in *Pneumocystis jirovecii* from Brazilian AIDS patients. J. Eukaryot. Microbiol. **53:**305–307.
47. **Worodria, W., M. Okot-Nwang, S. D. Yoo, and T. Aisu.** 2003. Causes of lower respiratory infection in HIV-infected Ugandan adults who are sputum AFB smear-negative. Int. J. Tuber. Lung Dis. **7:**117–123.
48. **Zar, H. J., M. J. Alvarez-Martinez, A. Harrison, and S. Meshnick.** 2004. Prevalence of dihydropteroate synthase mutants in HIV-infected South African children with *Pneumocystis jirovecii* pneumonia. Clin. Infect. Dis. **39:**1047–1051.
49. **Zar, H. J., A. Dechaboon, D. Hanslo, P. Apolles, K. G. Magnus, and G. Hussey.** 2000. *Pneumocystis carinii* pneumonia in South African children infected with human immunodeficiency virus. Pediatr. Infect. Dis. J. **19:**603–607.