Antiretroviral Drug Resistance Mutations Among Treated and Treatment-Naive Patients in Pakistan: Diversity of the HIV Type 1 *pol* Gene in Pakistan

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Abstract

Pakistan is experiencing a growing HIV epidemic. Antiretroviral drugs (ARV) have been smuggled into the country and available without prescription since the early 1990s, but are now provided free of cost by the government. We assessed the prevalence of HIV-1, drug resistance, and subtype distributions. Blood specimens were collected from HIV-1-infected participants registered in Sindh Province on dry blood spot (DBS) cards in 2008. *Pol*, protease, and partial reverse transcriptase regions were sequenced after reverse transcriptase PCR (RT-PCR). HIV-1 subtype was assigned by phylogenetic analysis. Primary drug resistance was analyzed by the Calibrated Population Resistance (CPR) tool using the Stanford Surveillance Drug Resistance Mutation (SDRM) major mutation list. Out of 100 blood samples collected, 42 were suitable for testing. Out of 42, 11 were ARV-receiving and 31 ARV-naive patients. Among them, 24 were injection drug users (IDUs), four immigrants, two hijras (male transvestites), two men who have sex with men (MSM), four prisoners, one female sex workers, two spouses of HIV-infected persons, and four from the general population. ARV resistance among naive patients was 2/31 (6.5%) and 36.4% (4/11) among ARV-experienced patients making an overall resistance of 14.2%. HIV-1 subtype A1 was the predominant subtype found in 35/42 (83.3%) followed by CRF35_AD and C, 6.5% each. Subtype D and G were found in one (2.4%) each. A significant proportion of Pakistani HIV patients has ARV drug resistance. Physicians treating patients should consider the magnitude of drug resistance while selecting regimens, and address drug adherence aggressively.

Introduction

PROGRESS IN ANTIRETROVIRAL THERAPY (ART) has resulted in dramatic achievements, such as the considerable reductions in morbidity and mortality observed in HIV-infected patients.¹ Antiretroviral (ARV) drug resistance is a fundamental survival strategy for the virus that stems from its vast capacity to generate diversity.² The overall prevalence of ARV resistance in the developing world, focusing on (1) treatment-naive populations, (2) the resistance consequences of prevention of mother-to-child transmission (PMTCT) drug regimens, and the relationship of medication adherence to resistance, has been described in a review of 117 sources.³ The

patterns of ARV resistance among treatment-naive populations worldwide appear to reflect geographic trends in use of ARV medications. A worldwide surveillance program (WATCH) found the rate of resistance (to any drug) among treatment-naive individuals was 5.5% in Africa, 7.4% in East Asia, 5.7% in Southeast Asia, and 6.4% in Latin America, lower than in North America (11.4%) and Europe (10.6%). Resistance data on HIV clades other than A, B, C, and D were too scarce to permit reliable conclusions.³

Pakistan is experiencing a concentrated epidemic among injection drug users (IDUs) and male sex workers (MSWs).^{4,5} Even though ARV drugs have been provided free of charge in Pakistan since 2004 through the National AIDS

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Control Program with support from the Global Fund to fight AIDS, Tuberculosis and Malaria, ART drugs have been smuggled into and available in Pakistan without prescription since the early 1990s. A few nongovernment organizations (NGOs) have been providing ARV drugs irregularly to HIV-positive persons.⁶ Selected pharmacies were also smuggling these drugs and gave ARV drugs as available to patients depending on their availability.⁶

As of February 2010, there were 1381 patients receiving ARV drugs in the country and 3176 were registered to receive them in due course of time (National AIDS Control Program, Ministry of Health). However, there are no systematically obtained data on ARV drug resistance in Pakistan and there are few studies on the distribution of HIV subtypes in the country. Local knowledge of resistance is necessary to guide testing and therapeutic choices. Moreover, the viral diversity has implications for possible differential rates of disease progression, responses to antiretroviral therapy (including the development of resistance), and vaccine development.⁷ We conducted the first study in Pakistan to assess the drug resistance and circulating subtypes of HIV-1 among treatment-experienced and treatment-naïve patients.

Materials and Methods

Study participants and specimen collection

A cross-sectional study was conducted at the ARV Center of Sindh AIDS Control Program in Karachi, Pakistan. We calculated a sample size of 105 and these study participants were selected using a simple random sampling technique from a pool of 560 HIV-infected persons seen between June and August 2008. Study participants included registered patients receiving ARVs and those not on ARVs whose CD4 cell count was >350. The field team (a research officer and two health workers) was trained for 2 days on interviewing, communication, consent taking procedures, maintaining privacy, and dry blood specimen (DBS) collection. The study participants were contacted by the field team and the objectives of the study were explained in detail. All study participants were recruited after obtaining informed consent. After completing the interview, a DBS sample was obtained. The questionnaire collected basic demographic information, history of injection drug use, history of work-

ing as a commercial sex worker, travel abroad for employment purposes, and HIV status of spouse. The study was reviewed by the ethical review committee of Bridge Consultants Foundation and Vanderbilt University Institutional Review Board. DBS were prepared according to a protocol described by Youngpairaj *et al.*⁸ Fifty microliters of whole blood was spotted on 903 filter paper (Schleicher & Schuell, Keene, NH) and dried at room temperature for 3 h. The DBS were then sent to the China Center for Disease Control and Prevention for further genotypic testing at room temperature.

Nucleic acid extraction, reverse transcriptase-polymerase chain reaction and sequencing

Total nucleic acids were extracted from DBS using a modified Nuclisens assay method (bioMérieux, Inc., Durham, NC). Partial HIV-1 *pol* gene was amplified by nested RT-PCR using an in-house method described by Liao *et al.*⁹ The whole protease (codons 1 to 99) and a part of the reverse transcriptase (RT) (codons 1 to 250) was sequenced bidirectly using the BigDye sequencing kit (Applied Biosystem, Foster City, CA).

Phylogenetic and drug resistance analyses

The *pol* sequences were aligned with reference sequences from the Los Alamos database (<http://hivweb.lanl.gov>). HIV-1 subtype was assigned by phylogenetic analysis. Sequences from drug-naïve individuals were analyzed for the transmitted drug resistance mutations with the Calibrated Population Resistance (CPR) tool available at <http://cpr.stanford.edu/cpr/servlet/CPR>. For ART-experienced patients, drug resistance mutations were identified and interpreted with the HIVdb program available at <http://sierra2.stanford.edu/sierra/servlet/JSierra>.

Results

Samples

We were able to recruit 100 ARV treatment-receiving and treatment-naïve patients from the 105 sampling frame. Three study participants cited shortness of time to participate in the research as the primary reason for refusal and two patients could not be traced by the study team. Out of the 100 samples,

TABLE 1. SUBTYPE DISTRIBUTION ANALYZED BASED ON HIV-1 1.2K SEQUENCE OF *pol* GENE

Subgroups	Subtype distribution					Total
	A1	AD	C	D	G	
Immigrants	1	1	1	1	0	4
Hijra (transgender)	2	0	0	0	0	2
Men who have sex with men	2	0	0	0	0	2
Injection drug users	24	0	0	0	0	24
Prisoners	3	1	0	0	0	4
Female sex workers	1	0	0	0	0	1
Spouses infected with HIV	2	0	0	1	0	3
General population	0	0	2	0	1	3
Total	35 (83.3%) ^a	2 (4.7%)	3 (7.1%)	2 (4.7%)	1 (2.3%)	42 (100%)

^aThe proportion of a specific subtype in total 42 samples.

Although A1 is the most prevalent (83.3%) genotype among HIV-positive persons in this study, AD, C, D, and G were also identified. We found a significant (21.2%) proportion of HIV-positive persons, those who were ARV naïve, infected by strains harboring mutations associated with ARV drug resistance. A previous study in 2006 analyzed HIV-1 subtypes among IDUs in Karachi and found subtype A in all the 34 samples.¹⁰ Earlier in 2000 we assessed gene sequences of 10 HIV-infected persons from Pakistan and found subtype

A among four, C in three, and D in three samples.¹¹ It is noteworthy that until 2003 a heterosexual mode of transmission was the predominant route of transmission in Pakistan.¹² However, the situation changed in 2004 following the outbreak of HIV among IDUs in a small town in Sindh Province

TABLE 2. PHENOTYPIC AND GENOTYPIC PATTERNS OF SAMPLES

Treatment	Number of specimen	Subtype	Point mutation		
			NRTIs	NNRTIs	PIs
On ART (N=11)	PA002	CRF35_AD	—	V90I	—
	PA008	B/C	D67G K70R M184V K219N	K103N Y181C G190A	—
	PA010	A1	—	—	—
	PA025	A1	—	E138A	—
	PA028	A1	—	E138A	—
	PA031	A1	—	E138A, F227FL	—
	PA043	A1	—	E138A	—
	PA045	A1	L210S	E138A, K101Q	—
	PA047	A1	—	E138A, K101Q	—
	PA080	A1	—	E138A	—
	PA094	C	—	—	—
Non-ART (N=31)	PA009	D	—	—	—
	PA012	A1	—	—	—
	PA014	A1	—	—	—
	PA016	A1	—	—	—
	PA020	A1	—	—	—
	PA022	A1	—	—	—
	PA023	A1	—	—	—
	PA029	A1	—	—	—
	PA032	A1	—	—	—
	PA035	A1	—	—	—
	PA041	A1	—	—	—
	PA054	A1	—	—	—
	PA057	A1	—	K101E	—
	PA058	A1	—	—	—
	PA059	A1	—	—	—
	PA060	A1	—	—	—
	PA062	A1	—	—	—
	PA063	A1	—	—	—
	PA064	A1	—	—	—
	PA066	A1	—	—	—
	PA067	A1	—	—	—
	PA068	A1	—	—	—
	PA069	A1	—	—	—
	PA070	A1	—	—	—
	PA073	A1	—	—	—
	PA074	A1	—	—	—
	PA076	CRF35_AD	—	—	—
	PA089	B/G	—	—	L90M
	PA090	A1	—	—	—
	PA091	A1	—	—	—
	PA095	C	—	—	—

All analyses for 47 serospecimens were performed using HIVdb Program Genotypic Resistance Interpretation Algorithm.

NRTIs, nucleoside reverse transcriptase inhibitors; NNRTIs, nonnucleoside reverse transcriptase inhibitors; PIs, protease inhibitors; ART, antiretroviral therapy; —, susceptible or no drug resistance mutations.

in 2003¹³ and injection drug use has now become the most common route of transmission (National AIDS Control Program).

Although subtype A was present in a previous Pakistani study,¹⁰ A1 may now be dominant (82.9%), probably because the mode of transmission has changed from sporadic heterosexual to endemic injection drug use. In the late 1990s, laborers working in the Gulf States were the largest number of reported HIV-positive persons; all of them acquired HIV while working out of the country and from fe-

male sex workers.¹⁴ Female sex workers from different parts of the world have been known to go to the Gulf States for commercial sex. Subtype AD, which is also a variant of A, has been reported from IDUs in Afghanistan and Iran in 2007,¹⁵ while two separate studies in Manipur have reported subtype C as the predominant subtype among IDUs in India.^{16,17}

ARV drug resistance rates were 7.4% in East Asia and 5.7% in Southeast Asia,³ but we found a higher rate (21.2%). This is still lower than neighboring India (Pune, Maharashtra), where

Sen *et al.*¹⁸ found at least one HIV drug-resistant mutation in RT in 29 (80.5%) ARV-experienced patients with evidence of failing therapy. M184V was the most commonly observed HIV drug-resistant mutation. No PR major mutations were observed among ARV-experienced patients. The same study also revealed that out of 75 HIV-1 sequences, 73 belonged to subtype C and 2 to subtype A1. One or more HIV-resistant mutations occurred in 81.8% of the 33 antiretroviral treatment-experienced study participants with evidence of virologic failure; M184V was the most commonly observed resistance mutation (69.7%) in 2007 from India.¹⁹ The plausible reason for lower ARV resistance mutation rates in Pakistan may be the late introduction of widespread ARV use compared to India. In our study at least three patients showed resistance to protease inhibitors (PI) and were possibly previously exposed to ARVs, as one of them showed drug resistance with a primary resistance mutation L90LM and two with secondary mutations E35EG and M46T, respectively. Another patient showed drug resistance to nonnucleoside reverse transcriptase inhibitors (NNRTIs), with two mutation points K101E and E138A.

There are certain limitations in our study. The sample size was smaller than expected as we were able to find only 47 samples suitable for testing out of 100. DBS is still a new technique in Pakistan and retraining of staff for correct and adequate collection is in progress. However, the samples are likely representative of all high-risk groups. We intend to conduct a larger study to determine mutation and drug resistance among ARV-experienced and ARV-naïve patients.

This is the first study reporting major mutations by genotyping in ARV-receiving and ARV-naïve patients from Pakistan; we suspect these mutations are likely to increase in the future. The findings also emphasize the need for resistance monitoring and use of optimal ARV combinations. HIV-1 drug resistance testing is recommended before starting treatment in order to achieve better clinical outcomes. HIV prevention remains a major priority to minimize the number of persons in Pakistan requiring ARV therapy.

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Author Disclosure Statement

No competing financial interests exist.

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