Genotype and antiretroviral drug resistance of human immunodeficiency virus-1 in Saudi Arabia

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ABSTRACT

الأهداف: تحليل مقاومة فيروس نقص المناعة المكتسب (HIV-1) لمضادات الفيروسات القهقرية وتحديد التنوع الجيني للفيروس لدى المرضى السعوديين وذلك بمعاينة التسلسل الجيني لمنطقة مضخمة من جين بول الفيروسي (pol gene).

الطريقة: أُجريت هذه الدراسة الاسترجاعية في وحدة الكائنات المعدية الخاصة بمركز الملك فهد للبحوث الطبية في جامعة الملك عبد العزيز، جدة، المملكة العربية السعودية وذلك خلال الفترة من أغسطس 2004م إلى يونيو 2009م. لقد تم تحليل عينات البلازما المأخوذة باستخدام نظام التعريف الجيني (Viroseq 2.5) من شركة (Celera / Abbott)، بالإضافة إلى استخدام المحلل الجيني (ABI Prism 3100) لتحديد التسلسل الجيني. لقد كان كل المرضى من الجنسية السعودية الذين قد تلقوا العلاج بمضادات الفيروسات القهقرية، غير أن بعضهم لم ينجح معهم العلاج.

النتائج: من بين 63 عينة تم تقسيم الأنواع الجينية اعتماداً على منطقة البروتيز (PR) إلى الأنواع التالية: C:22، G:21، B:9، CRF02_AG:5، D:3، A:1، F:1، J:1. فيما تم تقسيم الأنواع الجينية اعتماداً على منطقة الناسخ العكسي (RT) كالتالي: C:23، G:24، B:9، D:2، A:1، F:1 CRF02_AG:2. وأظهر اختبار استجابة العينات لمضادات الفيروسات القهقرية النتائج التالية: استجابت (52%) من العزلات للمجموعات الثلاثة الرئيسية من المضادات، واحتوت (41%) من العزلات على بعض الطفرات التي تسبب درجة عالية من المقاومة لواحد أو أكثر من مثبطات الناسخ العكسي الشبيهة بالنيوكلوسايد، فيما احتوت (16%) من العزلات على الطفرات التي تسبب درجة عالية من المقاومة لمثبطات الناسخ العكسي غير الشبيهة بالنيوكليوسايد، واحتوت (13%) من العزلات على الطفرات التي تسبب درجة عالية من المقاومة لواحد أو أكثر من مثبطات البروتييز. لقد استجابت أكثر العزلات لمجموعتين أو مجموعة واحدة على الأقل من مضادات الفيروسات القهقرية، غير أن 3% من العزلات فقط قامت بمقاومة العديد من أعضاء المجموعات الثلاث.

خاتمة: تشير الدراسة بأن مقاومة مضادات الفيروسات القهقرية تعد شائعة بين المرضى السعوديين الذين يتلقون العلاج بهذه المضادات.

Objectives: To analyze antiretroviral drug resistance and determine the genotype of human immunodeficiency virus (HIV)-1 in Saudi patients by sequencing an amplified region of the viral pol gene.

Methods: This retrospective study analyzed data from plasma samples submitted for genotypic drug sensitivity monitoring. Samples were analyzed at the Special Infectious Agent Unit, King Fahd Medical Research Center of King Abdulaziz University, Jeddah, Kingdom of Saudi Arabia from August 2004 to June 2009. The Viroseq2.5® kit (Celera/Abbott) was used with ABI Prism 3100 sequencer. All patients were Saudi nationals and were on antiretroviral therapy, some experiencing treatment failure.

Results: Based on protease region (PR), genotypes of 63 samples were as follows: C:22, G:21, B:9, CRF02_AG:5, D:3, A:1, F:1, and J:1. Based on reverse transcriptase region (RT), genotypes were as follows: C:23, G:24, B:9, CRF02_AG: 2, D:2, A:1, and F:1. Antiretroviral susceptibility testing results were as follows: 52% of the isolates were susceptible to all 3 major classes of antiretroviral drugs used, 41% had mutations known to confer high level resistance to one or more of the nucleoside analogue reverse transcriptase inhibitors, 16% had mutations known to confer high level resistance to non-nucleoside analogues reverse transcriptase inhibitors, 13% had mutations known to confer high level resistance to one or more of the protease inhibitors (PI). Most isolates were susceptible to 2 or at least one class of antiretroviral, and only 3% of the isolates had resistance to several members of all 3 classes.

Conclusion: Antiretroviral resistance is not uncommon in Saudi patients on antiretroviral therapy.

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Development of resistance to antiretroviral drugs constitutes a threat to patients undergoing treatment and a continuous concern to their doctors. The transmission of resistant viruses is also a concern to the community in general.[1] Drug resistance assays have become an important tool for the management of human immunodeficiency virus (HIV) infected subjects.[2-5] Many studies[6-11] have been carried on antiretroviral resistance in various countries. Reports on drug resistance from newly infected patients in Europe revealed resistance rates of 9-21%,[6-9] while a study from the USA[10] on newly infected patients indicated a rate of 12.4% phenotypic resistance, and a rate of resistance mutations (genotypic resistance) of 8-22.7%, while multi-drug resistance increased up to 10.2%. Another USA study[11] indicated that as many as 76% of viremic patients receiving antiretroviral therapy are infected with viruses that express resistance to at least one of the available antiretroviral drugs. Resistance to a drug was found to be associated with reduced responsiveness to the drug in vivo.[4,5] Two types of assays are used for antiretroviral susceptibility testing: phenotypic assays, which measure the susceptibility of the virus to various drugs in tissue-culture systems; and genotypic assays, which detect the presence of resistance mutations by sequence analysis of selected regions of the viral genome.[2,12] Both are used, each having certain advantages and disadvantages. Phenotypic assays provide quantitative resistance information and information on new drugs for which genetic correlates have not been established. However, they are more complex to perform, costly, and time consuming, which limits their availability. Genotypic assays are relatively easier to perform, have a quicker turnaround time, and are more widely available.[2] These assays usually involve amplification and sequencing of the reverse transcriptase (RT) and protease (PR) genes, and their analysis for known resistance mutations from widely used data banks. We aimed to shed light on the extent of HIV drug resistance in Saudi patients. Such information will be useful for guiding antiretroviral therapy. In addition, defining the most prevalent genotypes in Saudi Arabia would give us better insight on the source and transmission of HIV in this country.

Methods. All patients were Saudi nationals treated as inpatients or outpatients at the King Saud Hospital, Jeddah, and HIV/acquire immune deficiency syndrome (AIDS) outpatient clinic (no samples were received from expatriate patients). Patients had chronic HIV infection and were on antiretroviral therapy. Regimens of antiretroviral drugs used for all categories (adults, pregnant women, children, and neonates) were according to the current updates of the Guidelines of the Panels on Antiretroviral Therapy, Department of Health and Human Services (USA).[13-15] The backbone of therapy was 2 nucleoside analogue reverse transcriptase inhibitors (NRTIs) such as zidovudine, stavudine, or didanosine plus lamivudine, combined with a protease inhibitor (PI) usually lopinavir/ritonavir, atazanvir, or nelfinavir, or a non-nucleoside reverse transcriptase inhibitor (NNRTIs), such as efavirenz or nevirapine. Routine investigations for all patients before initiation of antiretroviral therapy (ART) include quantitative HIV polymerase chain reaction (PCR) (viral load). Patients receiving antiretroviral therapy for at least 6 months, and failing to achieve undetectable viral load were suspected to have antiretroviral resistance. Suboptimal response to treatment as judged clinically, and/or high or increased viral load were the main reasons for ordering drug resistance testing. Inclusion criteria for analyzed samples were Saudi nationals, and viral load >1000 copies of HIV ribonucleic acid (RNA)/ml. Samples with low copy number, as judged by electrophoretic band intensity upon amplification, and viral load were excluded from further analysis. Plasma samples from 63 patients were analyzed at the Special Infectious Agent Unit, King Fahd Medical Research Center (KFMRC) of King Abdulaziz University, Jeddah from August 2004 to June 2009. Ethical approval for the study was obtained from the Medical Ethics Committee at the Faculty of Medicine, King Abdulaziz University. The Viroseq2.5® kit (Celera/Abbott, Alameda, CA, USA) was used with the ABI Prism 3100 sequencer (Applied Biosystems, Carlsbad, CA, USA) for sequencing all isolates. Processing was according to the manufacturers instructions. Viral RNA was purified and reverse-transcribed into cDNA. A 1.8 kb fragment of the pol gene of the HIV-1 genome spanning the entire PR region, and approximately two-thirds of the RT region was amplified by the PCR. Within this fragment, a l.3 kb region was sequenced. This regions comprises the entire PR (codons 1-99), and a fragment (codons 1-335) of the RT. An ABI Prism 3100 (Applied Biosystems, Carlsbad, CA, USA) is a capillary-based automated fluorescent sequencer that relies on Sanger's method incorporating dye-labelled ddNTP terminators during DNA extension. The obtained sequence was compared on-line for resistance interpretation using the Stanford HIV Drug Resistance Report (http://hivdb.stanford.

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edu). Susceptibility results were reported for the 3 major classes of antiretroviral drugs, such as NRTI, NNRTI, and PI.

The Excel Program (Microsoft, Redmond, Washington, USA) was used for data handling and statistical analysis.

Results. The procedure used in this study for both genotyping and antiretroviral resistance determination is based on the amplification and sequencing of the whole PR gene, and two-thirds of the RT gene of HIV-1. These genes code for the 2 main enzymes that are targeted by most current antiretroviral drugs. Moreover, they each contain sufficient variability so as to be used for genotyping of various HIV-1 isolates. **Table 1** shows the genotypes among the 63 Saudi patients. As can be seen, genotypes C and G were most common constituting 33-38% each. Genotype B was less frequent (14%). Circulating recombinant form CRF02_AG was also detected at low frequency. These results indicate that Saudi patients were infected by several HIV-1 genotypes. Agreement between the PR-based and RT-based genotypes, which was observed in 57 (90.5%) isolates serves as a double-check of the genotyping results for both of these genes. However, there was mismatching in 6 (9.5%) isolates. **Table 2** shows the results of antiretroviral susceptibility testing. Susceptibility to all 3 categories of antiretrovirals was 52% for all isolates. Resistance to NRTI (41%) was most common perhaps reflecting the longer experience with the use of these drugs. Resistance to NNRTI (usually considered for the whole class of these drugs) was 16%, and resistance to PI was 13%. Resistance to 2 classes or even the 3 classes of antiretroviral drugs was also observed. Resistance to NRTI plus NNRTI amounted to 8%, while resistance to NRTI plus PI amounted to 14%. Thus, dual class resistance amounted to 22%. No simultaneous resistance to NNRTI and PI was observed Three percent of the isolates were simultaneously resistant to all 3 categories of antiretrovirals. **Table 3** lists antiretroviral drugs and well-known mutations that were detected in this study, which confer high-level resistance to each drug. Additional mutations, or combinations thereof are recognized to give intermediate, or low level resistance to particular drugs. Only mutations causing high level resistance are listed in this table. Those causing intermediate or low level resistance are too numerous to list. **Table 3** indicates the frequency (percentage) of high, intermediate, or low level resistance among our isolates based on mutations that they contain. Common mutations conferring high level resistance include: M184V, T215F/Y, M41L, K65R, T69D, K103N, V106A, Y181I, L90M, and I54V. Certain mutations, such as M184V confer resistance to several drugs particularly of the same class (such as, NRTI). For the 4 drugs, efavirenz, atazanavir, ritonavir, and saquinavir, high level resistance was due to the combination of mutations each recognized when present alone only to cause intermediate, or low level resistance. **Table 4** shows the frequency of high-level drug resistance mutations among the 63 samples analyzed. Thirty-three samples (52%) had no such mutations detected. **Table 5** lists high-level mutations encountered in individual patients on different antiretroviral regime. Most mutations encountered are well recognized for their occurrence during long-term antiretroviral therapy.

Discussion. Antiretroviral drug resistance testing is recommended in acute and early infection before initiation of therapy, in treatment initiation of chronic HIV infection, in all cases of treatment failure in patients on antiretroviral therapy, and in pregnancy.[1,2] Resistance testing may also be considered in other situations, such as for post-exposure prophylaxis where testing is carried

Table 1 - Number of HIV-1 PR-and RT-genotypes among 63 patients.*

Genotype	PR-based* genotype	(%)	RT-based* genotype	(%)
C	22	(35)	23	(36.5)
G	21	(33)	24	(38)
B	9	(14)	9	(14)
CRF02_AG	5	(8)	2	(3)
D	3	(5)	2	(3)
A	1	(1.5)	1	(1.5)
F	1	(1.5)	1	(1.5)
J	1	(1.5)		
Undetermined	-		1	(1.5)

*Total mismatches between PR and RT genotypes: 6, PR - protease gene, RT - reverse transcriptase (polymerase) gene, CRF - circulating recombinant form

Table 2 - Pattern of antiretroviral resistance.

Category	n	(%)
Susceptible to NRTI + NNRTI + PI	33	(52)
High-level resistance to at least one NRTI	26	(41)
High-level resistance to NNRTI	10	(16)
High-level resistance to at Least one PI	8	(13)
Resistance to NRTI + NNRTI	5	(8)
Resistance to NRTI + PI	9	(14)
Resistance to NNRTI + PI	–	–
Resistance to NRTI + NNRT+, PI	2	(3)

NRTI - nucleoside analogue reverse transcriptase inhibitors, NNRTI- non-nucleoside analogues reverse transcriptase inhibitors, PI - protease inhibitors

Table 3 - Resistance to individual antiretroviral drugs.

Anti-HIV agent	With high-level resistance (n)	(%)	Mutations	With intermediate-level resistance (n)	(%)	With low-level resistance (n)	(%)
3TC	23	(37)	M184V K65R	1	(2)	-----	---
ddC	----	---	K65R M184V	----		-----	---
AZT	10	(16)	T215F/Y K219E	5	(8)	2	(3)
ddI	4	(6)	T69D+ M184V	8	(13)	13	(20)
d4T	8	(13)	K70R+ M41L K70R T215Y	6	(10)	6	(10)
ABC	2	(3)	M184V K65R	11	(17)	15	(24)
TDF	---	---		6	(10)	9	(14)
FTC	19	(30)	M184V	----	---	1	(2)
DLV	8	(13)	K103NV106A, Y181I	1	(2)	3	(5)
EFV	7	(11)	Combination	1	(2)	3	(5)
ETR	----	---		1	(2)	2	(3)
NVP	9	(14)	V106A	6	(10)	3	(5)
ATV/r	1	(2)	Combination	11	(17)	11	(17)
DRV/r	----	--		----		4	(6)
FPV/r	1	(2)	I50V M46I	7	(11)	10	(16)
IDV	3	(5)	I54V L90M V82A	6	(10)	13	(20)
LPV/r	----	---		8	(13)	11	(17)
RTV	2	(3)	Combination	6	(10)	5	(8)
NFV	11	(17)	L90M D30N	11	(17)	3	(5)
SQV	2	(3)	Combination	11	(17)	10	(16)
TPV/r	----	---	----	2	(3)	6	(10)

3TC - Lamivudine, ddC - Zalcitabine, AZT - Zodovudine, ddI - Didanosine, d4T - Stavudine, ABC - Abacavir, TDF - Tenofovir, FTC - Emtricitabine, DLV - Delavirdine, EFV - Efavirenz, ETR - Etravirine, NVP - Nevirapine, ATV/r - Atazanavir+ritonavir, DRV/r - Darunavir+ritonavir, FPV/r - Fosamprenavir+ritonavir, IDV - Indinavir, LPV/r - Lopinavir+ritonavir, RTV - Ritonavir, NFV - Nelfinavir, SQV - Saquinavir, TPV/r - Tipranavir+ritonavir

out on the source of infection.[1,2] Resistance testing should help the clinician to avoid unnecessary switching of drugs, rule out adherence problems, perform well-directed switches rather than empirical changes of drugs, use active drugs for longer periods of time, save costs associated with switching drugs, and avoid unnecessary toxicities from inactive drugs.[2] Antiretroviral therapies with major RT and PI, as well as combination drugs have been available for a number of years in Saudi Arabia.[16] These drugs are given free of charge to Saudi nationals. It is expected under these circumstances that antiretroviral resistance will develop.[11]

The current study indicates that HIV resistance to any of the 3 major classes of drugs used in Saudi Arabia is significant, and must be taken into consideration in the treatment of chronic HIV infected patients. Furthermore, simultaneous resistance to 2 or all 3 classes of drugs is detected, further complicating therapy. As our study was conducted on chronically infected patients already on antiretroviral therapy, further studies on treatment naive patients are indicated. Some of the frequent mutations revealed in this study, which are associated with NRTI resistance are: M184V, which confers high-level resistance to lamivudine, it is the main mutation that is observed in most viruses resistant to treatment with this drug.[1,3] The M41L, T215Y, T215F, K70R confer high level resistance to zidovudine, and low level resistance to stavudine, didanosine, and abacavir.[1,3] The K65R mutation confers resistance to zalcitabine, abacavir, and tenofovir, but not by zidovudine. Several mutations observed are associated with NNRTI resistance. The K103N mutation is associated with resistance to efavirenz, delavirdine, and occasionally nevirapine; this mutation may confer high level in vitro resistance to

Table 4 - Frequency of high-level drug-resistance mutations.

Mutation	n	Frequency (%)*
Mutations associated with resistance to NRTI		
M184V	22	(35)
M184I	1	(1.5)
K65R	1	(1.5)
M41L	5	(8)
L210W	1	(1.5)
T215F/Y	14	(22)
K219E	1	(1.5)
K70R	3	(5)
D67N	3	(5)
T69D	5	(8)
Mutations associated with resistance to NNRTI		
K103N	8	(12.7)
V106A	1	(1.5)
Y188L	1	(1.5)
Y188I	1	(1.5)
K101E	1	(1.5)
Mutations associated with resistance to PI		
I54V	5	(8)
L90M	6	(9.5)
V82A	3	(5)
M46I	2	(3)
I50V	1	(1.5)
D30N	3	(5)
No mutations detected	33	(52)

*total number of patients - 63, NRTI - nucleoside analogue reverse transcriptase inhibitors, NNRTI - non-nucleoside analogues reverse transcriptase inhibitors, PI - protease inhibitors

Table 5 - Antiretroviral regimens in patients with high level resistance mutations.

Treatment regimen	n
AZT/ 3TC) + (LPV/r	
Mutations	
M184V + I54V	1
M184 + K65R + K103N	1
M184 + I54V + L90M	1
M184 + T215Y	1
M184V + I50V + I54V + M46I	1
AZT / 3TC) + NFV	
Mutations	
M184 + T215F + D30N	1
M184V	2
M184V +M46I	1
M184V +T69D + T215Y	1
M184V + T215F + K70R + Y181I	1
M184V + L90M	1
M184V + T215Y+ L90M	1
AZT / 3TC) + EFV	
Mutations	
M184 +T215F/Y + K219E + K103N	1
M184V + K103N	1
K103N	2
AZT / DDI + NFV	
Mutations	
M41L + L210W	1
K70R + D67N + K219E/Q	1
AZT / 3TC) + NVP	
M184V + T215Y +Y188L	1
(AZT / 3TC) + IDV	
M184V	2
(TDF/FTC) + EFV	
M184V+ K65R+K103N	1
AZT + DDI + EFV	
T215F + L90M	1
AZT + DDI + IDV	
M184V + T69D + M41L +I54V+ V82A	1
AZT + 3TC + IDV + RTV	
M184V + T215F + K70R + M41L + D67N + I54V + M46I +V82A + L90M	1

n - number out of the total 26 patients, AZT - Zidovudine, 3TC - Lamivudine, NFV - Nelfinavir,; EFV - Efavirenz, TDF - Tenofovir, NVP - Nevirapine, FTC - Emtricitabine, IDV - Indinavir, RTV - Ritonavir, LPV/r - Lopinavir +Ritonavir. Brackets indicate combined drug formulation

these drugs.[2] This explains the simultaneous resistance to NNRTIs observed. The Y181I and Y181C mutations are often associated with resistance to nevirapine. The V106A mutations accumulate during ineffective therapy with most NNRTI. The following observed mutations have been associated with resistance to PR inhibitors: L90M is observed during failure of therapy with most protease inhibitors; D30N is associated with nelfinavir resistance; I54V is frequently found after prolonged ineffective therapy with PI.

This study indicates that the most frequent HIV-1 genotypes are types C and G (35-38%), while type B only constituted 14%. Non-B subtypes are dominant in Africa and Asia. This favors an African or Asian source for most infections in Saudi patients. Genotype C of HIV-1 is the most frequent genotype in Africa and India, while genotype B is more prevalent in North America and Western Europe.[12,17,18] However, non-B genotypes have become increasingly more prevalent in some European countries making it more difficult to exclude these countries as a source of our isolates. The most prevalent HIV-1 genotypes globally in 1999 were genotypes C (56%), A (23%), B (8%), D (5%) and CRF01_AE (5%).[17] The CRF-01_AE is widely circulating in South East Asia.[18] The high percentage of genotype G in our findings was unexpected. In addition, no CRF-01_AE genotype was detected, while several CRF-02_AG isolates were found. It should be pointed out that genotyping based on pol gene sequences may differ from that based on env gene sequences as determined in the above-sited studies. The large African and Asian communities in Jeddah, and the possibility of transmission to nationals can explain the genotypic distribution observed in this study. Another factor in the transmission of non-genotype B is that, while B

genotype is dominant among homosexuals,[15] HIV is predominantly transmitted heterosexually in Saudi Arabia. [16]

The database available for comparison of new mutations, and which is used in this study is based on genotype B strains that are prevalent in the United States and Europe. This may cause overlooking some important differences in drug susceptibility with other genotypes. It has been reported that some genotypes of HIV-1 can be less susceptible to PI or NNRTIs than genotype B.[19] This includes genotypes C and G, which are quite prevalent in our population. Therefore, judgment must be exercised in adopting therapeutic regimens based on genotype B pattern of sensitivity. As some controversy remains on this issue, it is useful to collect additional data. This study was limited to Saudi nationals who only represent one fifth to one fourth the total HIV-infected population in Saudi Arabia, the rest being expatriates from various countries mainly in Africa, Asia, and the Middle East.[16] The study of resistance in the expatriate in comparison to the native population may add important information to drug resistance for all groups in this country. In addition, the rapidly changing dynamics of HIV resistance necessitate that resistance data be regularly updated. Unfortunately, this is restricted by the relatively high cost and technical requirements of the test. However, this may soon change with newer sequencing techniques that are becoming more widely available, giving substantial savings in cost and time.[20] Updating of resistance data in treatment-experienced patients, and the inclusion of treatment of native Saudi patients and expatriates must be considered in future studies.

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