Short term adherence tool predicts failure on second line protease inhibitor-based antiretroviral therapy

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Declaration

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DEDICATION

I would like to dedicate this MMED to my darling wife, Danielle who supported me through thick and thin in this project. Her constant love and support reminds me that anything is possible.
ABSTRACT

Background

The management of patients failing second line antiretroviral therapy (ART) is a critical area of study considering the increasing numbers of patients on second line regimens, and the expense and poor availability of third line ART. Most patients who experience virologic failure (VF) on second line ART in low-middle income countries fail due to poor adherence rather than antiretroviral resistance. Pharmacy refill is an easily implementable adherence measure which has shown to correlate with viral load monitoring and survival, and has potential over the short term to be used as a simple adherence tool to detect probable VF on second line ART. The benefit would be conservation of resources by rationally limiting need for viral load (VL) testing and, in those countries with access to third line ART, the need for resistance testing.

Methods

We conducted an observational cohort study of patients who initiated second line ART at the McCord hospital ART clinic, “Sinikithemba” in Kwazulu-Natal, South Africa. Using clinical and pharmacy refill data extracted from the clinic’s electronic database, we determined risk factors for VF. Three different methods of calculating short term pharmacy refill adherence were evaluated and compared with long term adherence after second line initiation. Different interval durations of short term pharmacy refill were also assessed to determine the optimum time period of pharmacy refill that correlates best with a virologic response on second line ART.

Results

We included 274 patients with a median follow up of 27 months on second line ART. The percentage of patients with VF ranged between 3% and 16%
at the end of each 6 month interval after initiating second line ART. 243 patients with at least one VL after 4 months on second line were analysed in the statistical analysis. Pharmacy refill assessed over shorter periods (4 to 6 months) correlated similarly with virologic suppression as pharmacy refill assessed over longer periods. The risk of VF fell 73% with each 10% increase in pharmacy refill adherence when adherence was assessed over a 4 month period. Low CD4 count at second line ART initiation was a significant independent risk factor for VF. A non-significant association was observed between longer duration on second line ART and risk of VF.

**Conclusion**

Patients identified as poorly adherent by short term pharmacy refill are at risk for VF on second line ART. This pragmatic adherence tool could assist clinical management by identifying patients who require adherence interventions early to prevent immunological and clinical failure. In addition, this short term adherence measure may assist in rationalizing the use of VL monitoring and in selecting out patients who may benefit from resistance testing.
ACKNOWLEDGMENTS

It has been a privilege working under the supervision of Professor Gary Maartens whose vision, guidance and patience have made this project possible. The genius of Dr Rory Leisegang formed the foundation of the statistical aspects of the study. Annemie Stewart’s contribution to the descriptive analysis, and encouragement are greatly appreciated. The data access and collection was supervised by Dr Henry Sunpath who assisted the data collectors, Philip Winternheimer and Mashuda Ally. Professor Richard Murphy provided access to an earlier dataset of the same cohort on which to build upon, and made a valuable contribution to the manuscript. Finally, I would like to acknowledge the staff of the McCord antiretroviral clinic, “Sinikithemba” who, for many years lead the fight against HIV/AIDS in Kwazulu-Natal, at the heart of the epidemic.
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(BioMed Central – Infectious Diseases)

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ACRONYMS AND ABBREVIATIONS

ART: antiretroviral therapy

CDC: Centre for Disease Control and Prevention

Clinical failure: Development of new WHO stage 4 defining conditions on ART

DRM: drug resistant mutations

EDM: electronic drug monitoring

GART: genotype antiretroviral resistance test

Immunologic failure: Decrease in CD4 by 50% or to pre-ART value

MPR: medication possession ratio

NGO: non-government organisation

NNRTI: non-nucleoside reverse transcriptase inhibitor

NRTI: nucleoside/nucleotide reverse transcriptase inhibitor

PI: protease inhibitor

Second line ART: lopinavir-ritonavir (or atazanavir-ritonavir) together with two NRTIs

TDM: therapeutic drug monitoring

VF: virologic failure: A plasma RNA viral load of >1000 copies/ml after a minimum of 6 months on second line ART

VL: viral load(s)

VS: virologic suppression

WHO: World Health Organisation (WHO)
PART A: STRUCTURED LITERATURE REVIEW

There is a growing number of South African patients receiving second line antiretroviral therapy (ART), which consists of lopinavir-ritonavir or atazanavir-ritonavir together with two nucleoside/nucleotide reverse transcriptase inhibitors (NRTIs). Approximately 500 000 people were estimated to be on second line ART worldwide in 2012 and this number is expected to increase exponentially. [1] In consequence, the numbers experiencing virologic failure (VF) on this regimen have increased and this risk of failure grows with time. Second line ART failure rates are reported in one review to be as high as 38% after 3 years.[2] Poor adherence is the most common cause of VF on second line ART [3–5] but DRM may be selected under drug pressure in a patient with suboptimal adherence.[6] Multiple viral mutations are required for high level PI resistance, unlike the first generation non-nucleoside reverse transcriptase inhibitors (NNRTIs) used in South Africa (Efavirenz/Nevirapine), which typically develop high level resistance with only a single mutation.[7] Consequently, protease inhibitors (PIs) are considered to be robust in comparison with NNRTIs.

Third line ART regimens have recently been made available in South Africa, although there are no clear guidelines from WHO regarding the use of these new drugs. of.[8] Understanding reasons for second line VF and identifying which patients are at risk of developing VF is a critical area of research as it directly impacts on the management of these patients. The genotype antiretroviral resistance test (GART) to determine the presence of drug resistance mutations (DRM) costs about R3000 ($300),[9] which is expensive for routine use given the scale of South Africa’s ART programme. A clear and cost-effective approach is needed to identify patients at risk of VF on second line ART as well as the likelihood of DRM in established second line VF. A recent local study suggested using serum and hair therapeutic drug monitoring (TDM) to select patients in VF on second line ART who may benefit from GART.[10]
The natural progression is poor adherence which results in VF, followed by immunologic failure and then finally clinical failure. It therefore makes sense to implement an adherence measure to detect a patient at risk of VF so that adherence issues can be addressed early rather than waiting for a high viral load (VL) or a dropping CD4 count. In this way, timely interventions may also limit VL and GART testing and reduce the costs of more expensive regimens.

Pharmacy refill is an adherence measure where actual pharmacy refill dates are compared with expected dates of refill. Determination of pharmacy refill adherence may be implemented in public sector ART clinics, especially in those where pharmacies are electronically recording dispensing. Pharmacy refill is affordable and easily implementable and has the potential to be used as an adherence tool to assist with the clinical management of patients on second line ART.

This literature review is in two parts. The first part describes studies which identify predictors of VF or virologic suppression (VS) in patients on second line ART. We identified 11 peer reviewed observational studies of adult patients describing factors associated with virologic outcomes on second line ART, predominantly in low-middle income countries. The second part describes studies illustrating the utility of pharmacy refill as an adherence measure and defining its association with virologic and immunologic response, survival and CD4 count monitoring. Of particular importance to our study is the potential for pharmacy refill to be used as an adherence tool over short periods to detect and predict VF. The highly cited work of Gross et al [11,12] was used as a starting point for the literature review on short term pharmacy refill and related references were taken from his papers. Some literature which describes the advantages and disadvantages of other adherence measures which may be used to assess adherence has also been included. Our study is one of few which describes adherence in a cohort on second line ART in a low-middle income country.
Part 1:
Studies identifying predictors of virologic outcome on second line

Tables 1a and 1b illustrate the included studies. Nine of the eleven studies reviewed were performed in low-middle income countries, including South Africa, and the remaining two in high income settings, namely Europe [13] and the United States of America (USA).[14] The findings of Napravnik et al in the USA [14] should be interpreted with caution as about half the patients in the cohort were started on a PI as first line therapy and changed to either an NNRTI, integrase inhibitor or second generation PI as second line. Only eight studies (Table 1b) examined adherence as a possible predictor of virologic outcome. Six of the eight measured adherence using self report or pill counts, which have been shown to overestimate adherence.[15] A previous study, using an earlier cohort from the same clinic as our study, used pharmacy refill as a measure of adherence, but this was calculated over the total period since second line initiation. [16] Pharmacy refill measured over the short term is more pragmatic as it requires less information to calculate and provides insight into recent adherence trends.

Adherence has been shown to be a significant determinant of virologic outcome on second line ART. [16–20] A lower CD4 count at second line switch was also found in several studies to be associated with a poor virologic outcome.[13,14,18,19,21] In addition, a higher VL at second line initiation has shown to either predict VF [13] or failure to achieve VS.[21] Patients who delay switching to second line ART after confirmed VF on first line are also at risk of subsequent VF on second line ART [13,22] Fox et al [22] found that patients changed to second line ART for reasons other than poor adherence were more likely to achieve VS. Murphy et al [16] found a non-significant association between the final six months of adherence measured by pharmacy refill on first line ART with ≥90% adherence in the first 12 months of second line ART (OR: 2.5; 95% CI 0.7-8.6; p 0.15). Pujades-Rodriguez et al [18] found, in a large multi-centre study in Africa and
Asia, that changing one NRTI instead of two at second line switch was associated with VF on second line ART. Furthermore, El-Khatib et al [5] found that the lack of a refrigerator at home was also associated with VF on second line ART, but during the period of study, lopinavir-ritonavir was dispensed as Kaletra® capsules, which were not heat stable. Alluvia®, which is a non-refrigerated lopinavir-ritonavir co-formulation was introduced in South Africa in June 2008.

In summary, factors associated with VF on second line ART include the presence of stage 4 conditions and a lower CD4 count at second line initiation. Patients who take a longer time to switch to second line ART on a failing first line regimen as well as patients who demonstrate poor adherence on second line ART are at risk of VF. Regimen potency is an additional factor that may be associated with virologic outcome on second line ART. There is a need for further studies in low-middle income countries with good adherence data and longer follow up durations, which will assist clinicians at the point of care to detect patients at risk of developing VF on second line ART.

**Part 2:**

**Utility of pharmacy refill as an adherence measure**

Adherence at ART clinics has traditionally been assessed by self-reports or pill counts, but these have shown to overestimate adherence.(15) Electronic drug monitoring (EDM) has been shown to correlate well with VS [24] but is expensive and requires active participation by the patient to ensure that adherence is recorded correctly[15] The EDM instrument is bulky and needs to be brought to each clinic visit so that the adherence data can be electronically downloaded. EDM is also not suitable for patients who use adherence aids such as pillboxes. Differentiating periods of non-use (e.g. hospitalisation, incarceration) from periods of non-adherence can be difficult. In addition, EDM does not provide information regarding pill-taking behaviour and is best used when combined with other adherence measures.[25] TDM is a direct adherence measure but is expensive and has limited availability.
Random serum TDM only provides a “snapshot” of a patient’s recent adherence as the plasma half-life of lopinavir in the currently used lopinavir/ritonavir formulation is 5-6 hours.[10] TDM may also overestimate adherence if a patient only takes his/her pills in the days leading up to the clinic visit – so called “white coat adherence”. [26] Non-standard procedures for collecting, testing and interpretation also remain a concern. [15]

Pharmacy refill is an adherence measure which has been suggested as an appropriate adherence measure in low-middle income settings. [27] There are several different methods to calculate pharmacy refill adherence. A medication possession ratio (MPR) is a measure of pharmacy refill which describes a patient’s drug exposure and has traditionally been described over the long term since regimen initiation. An MPR may be calculated by dividing the number of days of medication supplied since the patient started the regimen (numerator) by the number of days in the same time period (denominator) and multiplied by 100 to obtain an overall percentage adherence. [28] A cruder method of calculating an MPR is by dividing the number of refills since regimen initiation by the number of months in the same time period. [14] MPRs account for oversupply as there may be more days/refills of tablets dispensed than days/months in the time period resulting in an MPR of >1. MPRs over shorter time periods are advantageous from a clinical management point of view. [29] Short term refill data provides enhanced insight into recent adherence trends and is also easier to calculate as less refill information is required. A prescription-based measure of adherence, which is an indirect measure of pharmacy refill, was recently used in a large study of 1632 patients in the UK, [30] where short term adherence over 6 months was shown to correlate with the occurrence of viral rebound (9% decrease in viral rebound risk for every 10% increase in adherence (RR: 0.91; 95%CI, 0.87-0.95).

Pharmacy refill has been shown in numerous studies to correlate with VS, [27,31–34] immunologic outcomes, [35] development of DRM [36], and survival [37,38], and is increasingly being used in ART clinics in low-middle income countries as a cost-effective and easily implementable adherence
measure. Advantages include being obtainable from electronic records and being less susceptible to deception. The disadvantages of pharmacy refill data are that it cannot monitor pill-taking behaviour and assumes that patients have only one source for their medications.[15] Pharmacy refill may also not be useful if refills are sent automatically and may not be appropriate for patients who receive several months of medication at regular intervals.[15]

Nachega et al [33] conducted a retrospective observational cohort study on 2821 ART naive patients >18 years of age starting an NNRTI-based regimen between January 1998 and December 2003 from Aid for AIDS (AFA), a Southern African private sector disease management programme serving nine countries in Southern Africa. Adherence was assessed using monthly pharmacy refill claims since regimen initiation. Each 10% increase in pharmacy refill claim adherence greater than 50% correlated with a mean absolute increase of 0.10 in the proportion of patients with sustained VS. The association of pharmacy refill using MPR with VF was also demonstrated in a sub-analysis of 79 patients who initiated second line therapy in Peru.[39] For every 10% increase in MPR, there was an associated decrease in hazard ratio for VF on second line ART (HR=0.88; 95 CI, 0.77-0.99).

Grossberg et al [31] also demonstrated that pharmacy refill is associated with VL change and showed self reported adherence to be less sensitive to detecting poor adherence than pharmacy refill data. 110 HIV positive patients were enrolled from a Veterans’ Affairs Medical Centre (VAMC) in Philadelphia, Pennsylvania, USA in an observational cohort study. Each patient completed a self report adherence questionnaire and pharmacy refill data was extracted from the centre’s electronic database over the three month period prior to each VL taken. Only the pharmacy refill measure was significantly associated with VL change. The VL decreased by 0.12 log_{10} copies/ml for each 10% increase in pharmacy-based refill defined adherence compared with 0.05 log_{10} copies/ml for each 10% increase in self reported adherence.
Pharmacy refill data has also been shown to correlate well with survival. Nachega et al [38] analysed the outcome of more than 6000 ART naive patients > 18 years of age who initiated first line ART in the AFA programme. Patients with <80% adherence determined by pharmacy refill had a three times decreased survival compared with those who had >80% adherence after adjusting for baseline variables. (HR:3.23; 95%CI, 2.37-4.39; P<0.001).

Pharmacy refill data has also shown to be more accurate than the use of CD4 count monitoring (which is recommended by WHO as an alternative measure to identify treatment failure), [8] to detect patients in VF in resource limited settings. Bisson et al [40] conducted an observational cohort study on 1982 adult patients who initiated an NNRTI based regimen in the AFA programme. The primary endpoint was VF defined as >1000 copies/ml at six or twelve months after ART initiation or rebound viraemia (>1000 copies/ml) after an initial viral suppression of <400 copies/ml. Pharmacy refill adherence was calculated by dividing the number of refill claims since ART initiation by the number of months between ART initiation and the primary endpoint. Pharmacy refill outperformed CD4 count measurements in the ability to detect patients in current VF in the first year following ART initiation. Furthermore, pharmacy refill performed equally with CD4 count monitoring to detect breakthrough viraemia. In addition, pharmacy refill measurements recorded three months before a VL was taken were equally as effective as CD4 count monitoring between initiation and the time a VL was taken to detect VF. This suggests that short term pharmacy refill data is a promising alternative to detect current as well as future VF.

The time period of pharmacy refill that is best associated with virologic change was studied by Acri et al [11] who showed that refill adherence measured a minimum of 90 days before a VL is taken, correlates best with VL change. 110 patients who had been on ART for a minimum of three months were recruited from the VAMC in Philadelphia, USA. The four most recent refills of the index drug (PI, NNRTI or abacavir) were obtained from the electronic database. The dates of pharmacy refills over the 90 day supply preceding the date of participant enrolment were used to define the
adherence intervals (30, 60 or 90 days). An MPR was calculated by dividing the number of days supply by the number of days between refills and multiplying by 100 for each of the supply intervals. Each supply interval was then correlated with a log change in VL. This study also showed that shorter durations of pharmacy refill (e.g. 30 days) correlate well with a change in VL. However, only the 30 day interval beginning a minimum of 90 days prior to the VL of interest correlated with VL change. 30 day refills taken more proximally to a VL are poorly associated with VL change and could over-call imperfect adherence leading to unnecessary adherence interventions. Although this study was limited by a small sample size, the ability of short term pharmacy refill to predict VF has been validated in larger cohorts on first line therapy.[12]

In conclusion, it has been shown that short term pharmacy refill data on first line therapy can be used to predict VF. This has not been shown in patients on second line ART in a low-middle income setting. Using a cohort on second line ART at an ART clinic in Kwazulu-Natal, South Africa, we have studied risk factors for VF and correlated differing measures and durations of pharmacy refill extracted from the clinic’s electronic database with virologic outcome, in order to create a pragmatic adherence tool for use in ART clinics.
Table 1a: Studies describing factors associated with virologic suppression (VS) or virologic failure (VF) on second line (adherence not measured)

<table>
<thead>
<tr>
<th>Ref</th>
<th>Cohort</th>
<th>VS/VF rate</th>
<th>Significant factors</th>
<th></th>
</tr>
</thead>
</table>
| (22) | 328    | VS at 1 yr: 203/262 (77%) | ➢ Patients switched to second line before awaiting 2 high VLs  
➢ Second line switch for reasons other than poor first line adherence | HR:1.9  
95% CI 1.08 - 2.61  
HR:1.83  
95% CI 1.14 - 2.93 |
| (23) | 210 (39 children) | VS at 1 yr: 78/128 (60.9%) | ➢ No significant associated factors on multi-variate analysis |   |
| (13) | 2042   | (triple class VF over 5 yrs): 575/2042 (28.2%) | ➢ Lower CD4 at second line switch (<50)  
➢ Higher VL at second line switch (VL log10 ≥6)  
➢ VF rates lower in homosexual men  
➢ VF rates lower in patients who spent less time with a high VL on first line awaiting second line switch (3-6 months vs 0-3 months) | HR 1.70  
95% CI 1.38 - 2.11  
HR 1.86  
95% CI 1.30 - 2.67  
HR: 1; reference  
aHR: 1.43  
95% CI 1.11 - 1.84 |
| (14) | 488    | VF at 6 months: 12% (detectable VL at switch); 17% (undetectable VL at switch) | ➢ Lower CD4 count at second line switch  
➢ Lower VF rates in more recent calendar years (2008-2010 vs 1996-1998) | Relative HR 0.82  
95% CI 0.75 - 0.8  
Relative HR: 0.40  
95% CI 0.15 - 1.00 |
### Table 1b:
Studies describing factors associated with virologic suppression (VS) or virologic failure (VF) on second line (adherence measured)

<table>
<thead>
<tr>
<th>Ref</th>
<th>Pat. No</th>
<th>Adherence measure</th>
<th>VS/VF rate</th>
<th>Factors associated with VF/VS</th>
</tr>
</thead>
</table>
| (17) | 243     | Self report       | VF at 1 yr (9.6%) | - 30 day adherence <95% OR: 2.90 95% CI 1.12 - 7.54  
                                       |                     |                   |                           | OR: 0.94 95% CI 0.90 - 0.98  
                                       |                     |                   |                           | OR: 2.44 95% CI 0.97 - 6.17  |
| [21] * | 205 (417) | Self report; Clinician reported adherence | VS at 15 months: 98 (48%) | - Younger age (per 5 year increase) aRR: 0.89 95% CI 0.79 - 0.95  
                                       |                     |                   |                           | aRR: 1.59 95% CI 1.09 - 2.34  |
| **   | 212 (417) | Self report; Clinician reported adherence | VS at 15 months: 152 (72%) | - Shorter duration of viraemia on first line ART (<12 months vs >12 months) aRR: 1.22 95% CI 1.03 - 1.44  
                                       |                     |                   |                           | aRR: 1.16 95% CI 0.88 - 1.52  
                                       |                     |                   |                           | aRR: 1.33 95% CI 1.11 - 1.61  |
| [5]  | 115     | Self report       | VF 37 (33%) | - Attending a public vs an NGO clinic OR: 4.60 95% CI 1.8 - 11.3  
                                       |                     |                   |                           | OR: 6.7 95% CI 1.2 - 37.5  
                                       |                     |                   |                           | - Not having a refrigerator at home  
                                       |                     |                   |                           |  
                                       |                     |                   |                           |  

*OR: odds ratio, CI: confidence interval, aRR: adjusted risk ratio
<table>
<thead>
<tr>
<th>Reference</th>
<th>Number</th>
<th>Description</th>
<th>Follow-Up Interval</th>
<th>Findings</th>
<th>Odds Ratio</th>
<th>95% CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>[16]</td>
<td>165</td>
<td>Long term pharmacy refill (MPR) since second line initiation</td>
<td>VF: +/-25% at each 6 month follow up</td>
<td>Poor adherence only significant risk factor for VF</td>
<td>OR: 2.5 per 10% increase adherence</td>
<td>95% CI 1.3 - 4.8</td>
</tr>
<tr>
<td>[18]</td>
<td>632</td>
<td>Adherence index (AI): percentage of clinic visits attended without delay</td>
<td>VF at 6 months: 119/632 (18.8%)</td>
<td>Lower AI (383.5 vs 176 per 1000 person yrs; &lt;80% vs ≥ 95%)</td>
<td>IRR: 3.14</td>
<td>95% CI 1.67 - 5.90</td>
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<td></td>
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<td></td>
<td></td>
<td>changing 1 NRTI instead of 2 at second line start (179.2 vs 251.6 per 1000 person yrs)</td>
<td>IRR: 0.64</td>
<td>95% CI 0.42 - 0.96</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>lower CD4 at second line switch (&lt;50 vs &gt;200: 289.5 vs 173.6 per 1000 person yrs)</td>
<td>IRR: 1.61</td>
<td>95% CI 1.01 - 2.57</td>
</tr>
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<td></td>
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<td></td>
<td></td>
<td>Higher VF rates in 6-17 months after second line start vs ≥ 18 months (250.0 vs 123.2 per 1000 person years)</td>
<td>IRR: 1.90</td>
<td>95% CI 1.19 - 3.02</td>
</tr>
<tr>
<td>[19]</td>
<td>95</td>
<td>Self reports and pill counts</td>
<td>VS by 6 months: 54/95 (67%)</td>
<td>Higher adherence</td>
<td>HR: 2.94</td>
<td>95% CI 1.60 - 5.39</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>lower CD4 at second line start</td>
<td>HR: 1.13</td>
<td>95% CI 1.03 - 1.24</td>
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<td></td>
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<td></td>
<td></td>
<td>lower CDC classification at second line switch (A vs C)</td>
<td>HR: 3.11</td>
<td>95% CI 1.48 - 6.55</td>
</tr>
<tr>
<td>[20]</td>
<td>101</td>
<td>Self report and pill count</td>
<td>VS at 1 year: 86/101 (85.2%)</td>
<td>Adherence only</td>
<td>OR: 5.70</td>
<td>95% CI 1.16 - 27.93</td>
</tr>
</tbody>
</table>

HR: Hazard ratio; aHR: Adjusted hazard ratio; OR: Odds ratio; aRR: Adjusted relative risk ratio; IRR: Incidence rate ratio; * workplace programme; **community programme
References


PART B: JOURNAL READY MANUSCRIPT

Short term adherence tool predicts failure on second line protease inhibitor-based antiretroviral therapy: An observational cohort study

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ABSTRACT

Background

Most patients who experience virologic failure (VF) on second line antiretroviral therapy (ART) in low-middle income countries fail due to poor adherence rather than antiretroviral resistance. A simple adherence tool designed to detect VF would conserve resources by rationally limiting need for viral load (VL) testing and, in those countries with access to third line ART, the need for resistance testing.

Methods

We conducted an observational cohort study of patients who initiated second line ART at a clinic in Kwazulu-Natal, South Africa. Using clinical and pharmacy refill data extracted from the clinic's electronic database, we determined risk factors for VF. Three different methods of calculating short term pharmacy refill adherence were evaluated and compared with long term adherence since second line initiation. We also explored the ability of differing durations of short term pharmacy refill to predict VF on second line ART.

Results

We included 274 patients with a median follow up of 27 months on second line ART. VF ranged between 3% and 16% at the end of each six month period after initiating second line ART. 243 patients with at least one VL after
4 months on second line were analysed in the statistical analysis. Pharmacy refill adherence assessed over shorter periods (4 to 6 months) predicted virologic suppression as well as pharmacy refill assessed over longer periods. The risk of VF fell 73% with each 10% increase in adherence measured from pharmacy refills over a 4 month period. Low CD4 count at second line ART initiation was a significant independent risk factor for VF.

**Conclusion**

Patients identified as poorly adherent by short term pharmacy refill are at risk for VF on second line ART. This pragmatic adherence tool could assist in identifying patients who require adherence interventions, and help rationalize use of VL monitoring and resistance testing among patients on second line ART.

**Keywords**

**HIV**

**Second line antiretroviral therapy**

**Medication adherence**

**Virologic Failure**

**Pharmacy refill**
BACKGROUND

Approximately 500,000 people in low-middle income countries were estimated to be on second line protease inhibitor (PI)-based ART in 2012, and this number is expected to increase exponentially.[1] A systematic review reported virologic failure (VF) rates on second line ART in resource-limited settings to be as high as 38% after 3 years.[2] Risk factors for VF on second line ART include a lower CD4 count and the presence of WHO clinical stage 4 conditions at second line switch,[3–6], a longer duration between confirmed VF on first line and initiating second line, [7] changing one nucleoside/nucleotide reverse transcriptase inhibitor (NRTI) instead of two at second line ART initiation,[8] and poor adherence on second line ART.[9] The majority of patients with VF on second line ART have no major PI mutations, indicating that VF in most cases is not the result of antiretroviral resistance.[10–12] Identifying which patients on second line ART are at risk of developing VF will allow earlier implementation of adherence interventions and may rationalize the use of VL monitoring, which is not widely available in many low-income countries. The development of policies for a third line ART regimen is regarded as a priority by the World Health Organization (WHO).[13] Empirically switching patients failing second line ART to third line ART, which is currently expensive, would be a waste of resources as most patients do not have antiretroviral resistance. Switching to third line ART should ideally be guided by genotype antiretroviral resistance testing (GART), but this is currently expensive and has little availability in
resource limited settings. A simple predictor of VF on second line ART could also be useful in selecting patients who may benefit from GART.

Pharmacy refill is an adherence measure, which has been shown to correlate well with survival and virologic suppression.[14–16] A recent study from our group (of an earlier cohort from the same clinic from which the cohort used in the current study was obtained) showed that adherence measured by pharmacy refill correlated with virologic suppression on second line ART, but this was based on average adherence since starting second line.[9] A more pragmatic measure would be to assess adherence over the short term, which has been shown to predict VF in a small study in a high income country setting.[17]

We investigated the association between short term adherence, assessed by pharmacy refill, and VF in patients on second line ART at a clinic in Kwazulu-Natal, South Africa, in order to develop a clinical tool for use in ART programmes in low-middle income settings.

**METHODS**

**Study Population and Setting**

The McCord Hospital ART clinic, “Sinikithemba” meaning in Zulu “we give hope”, provided HIV care for patients from Durban and surrounding areas in Kwazulu-Natal, South Africa with financial support from the President’s Emergency Plan for AIDS Relief and the South African Department of Health. Approximately 8200 adults and 1200 children received ART at the
clinic until its closure in February 2012. The clinic followed national recommendations, which at the time included VL and CD4 count monitoring six-monthly after ART initiation. During the period of study, the standard South African second line regimen consisted of lopinavir/ritonavir with two NRTIs. As per WHO guidelines, second line initiation occurred either for toxicity/intolerability of first line drugs or for confirmed VF on first line ART.[13] Patients identified with VF on second line ART (>1000 copies/ml) after a minimum of six months of therapy, were referred for adherence counselling with a repeat VL measurement after three months.

Inclusion and exclusion criteria

We identified HIV-infected adults over 18 years of age who initiated second line ART following VF on first line ART between August 2003 and June 2011. Patients who switched to second line ART for reasons other than VF on first line were excluded. In an exploratory analysis we discovered several patients with suppressed VL despite zero adherence, indicating that they were obtaining ART from another site. For the statistical analysis, we therefore excluded patients with suppressed VL following a four month period of null adherence.

Clinical and demographic data was extracted from the clinic’s electronic database. including age, sex, CD4 and VL responses, and duration on second line ART. The date of each pharmacy refill as well as the number of pills dispensed was also recorded. A 30 day supply of medication was routinely dispensed with pharmacy refills scheduled after 28 days. Stable patients with virologic suppression were occasionally given 60 or 90 days of
medication with an appropriate follow up interval. Missed pharmacy refill dates resulted in a reminder telephone call from the clinic.

Study design

We conducted a retrospective observational cohort study of patients who initiated second line ART after confirmed VF on first line. We analysed the relationship between short term adherence measured by pharmacy refill and virologic suppression. We identified factors associated with VF and evaluated three different short term measures of pharmacy refill adherence and compared them with long term adherence measured from second line ART initiation. In addition, we explored the optimum duration of short term refill which correlated best with a virologic response.

Statistical Analysis

We performed all statistical analyses in Stata version 13. The pharmacy refill period before each VL (after a minimum of 4 months on second line ART) was used to analyse the relationship between pharmacy refill adherence and virologic suppression. We ensured that our method accommodated patients who had collected more than 30 days of medication at a single visit within the period being assessed. The association between pharmacy refill adherence and virologic suppression was evaluated using Receiver Operator Characteristics (ROC). We compared several methods of calculating adherence from pharmacy refill data over differing periods. We assessed both short term (from 3 to 12 months) and long term pharmacy refill (from second line initiation). We truncated adherence over 100% for all methods.
Short term adherence was expressed as a percentage and calculated using three different methods, termed “interval gap”, “interval average” and “interval crude”. “Interval crude”, was calculated by dividing the number of pharmacy refills within the interval (numerator); by the number of months within the interval (denominator). “Interval average”, which is similar to a method published in a previous study on short term pharmacy refill adherence,[15] was calculated as follows: number of days of medication dispensed within the interval, plus the accrued days of medication from the last dispensing event prior to the interval, less the unused days of medication from the last dispensing event within the interval (numerator); divided by the number of days with the interval (denominator). “Interval gap” is a new short term pharmacy refill adherence measure we created to account more accurately for gaps in medication days. The “interval average” method may over-estimate adherence, because patients receive 30 days’ supply at scheduled dispensing events every 28 days, therefore, if patients have a gap without medication but then attend subsequent scheduled dispensing events, the accrued two day supply for each dispensing event within the interval will be included in the numerator, reducing the days of medication missed. For each day within the interval, we determined whether the patients had medication according to the pharmacy refill data, allowing accrued tablets from the last dispensing event prior to the interval as with the “interval average” method. “Interval gap” was calculated as follows: the number of days within the period, less the number of days that the patient did not have medication (numerator); divided by the number of days within the period (denominator). Long term “overall” adherence was determined by dividing
the total number of days of medication dispensed since second line initiation (numerator), by the total number of days since second line initiation prior to the VL of interest (denominator).

Associations between virologic suppression and the different adherence measures were determined using the area under the curve derived from receiver operating characteristic (ROC) analyses, assessed over differing interval durations prior to a VL of 3 to 12 months. The best performing short term adherence measure and interval duration prior to a VL was then selected for subsequent analyses. We determined the association between virologic suppression and the identified variables in a multivariate logistic regression model. The variables included age, sex, CD4 and log_{10} VL at second line initiation, duration on second line, and adherence measured by short term pharmacy refill prior to a VL. Missing CD4 and VL values at second line initiation were imputed. The square root of the CD4 was used to attenuate the effect of higher values.

**Ethics**

This study was reviewed and approved by the University of Cape Town Human Research Ethics Committee and the McCord Research Ethics Committee.

**RESULTS**

Two hundred and ninety one patients met the inclusion criteria for the study, but there was missing data in 17 patients, who were excluded. The baseline
characteristics of the 274 included patients are shown in table 1. The proportions of patients with virologic suppression and VF over time on second line are shown in table 2. For the subsequent analyses, we excluded 21 patients who had no VL data ≥4 months after starting second line and a further 10 patients who had suppressed VL following a 4 month period of zero adherence, leaving 243 patients.

Adherence measured by the “interval gap” method out-performed both the “interval average” and “interval crude” methods (figure 1). Adherence measured by the “interval gap” method performed similarly over all of the interval durations assessed, with overlapping 95% confidence intervals of the ROC area under the curve. We chose 4 months of adherence measured by the “interval gap” method for subsequent analyses as a pragmatic time period to implement in clinics, considering that achievement of virologic suppression on a new regimen is usually attained by 4 months. Short term pharmacy refill measured 4 months prior to a VL predicted virologic response, with higher rates of adherence achieving superior virologic suppression (figure 2). Adherence measured by the “interval gap” method was equivalent to long term “overall” adherence at predicting virologic suppression (<400 copies/ml) – figure 3 (addendum).

Significant risk factors for VF on multivariate analysis were poor adherence measured by 4 months of “interval gap” pharmacy refill proximal to a VL and a lower CD4 count at second line initiation (table 3).
DISCUSSION

We have demonstrated that short term adherence measured by pharmacy refill was the strongest predictor of VF on second line ART. Short term pharmacy refill adherence was also associated with virologic suppression with an “adherence dose response” relationship. Our study is one of few which have evaluated adherence on second line ART and is novel in that adherence was measured over the short term. We found that the “interval gap” method, which is a method not previously used for calculating ART adherence from pharmacy refills, outperformed the usual methods that average adherence over an interval. We observed a trend towards an association between longer duration of second line ART and risk for VF, but this was not statistically significant. We found an association between lower CD4 count at the time of starting second line ART and VF on second line ART, which adds support to data showing that second line outcomes are improved with early detection of failure of first line ART and prompt initiation of second line ART before immunological deterioration.[18]

A previous report of an earlier cohort from the same clinic as the present study,[9] reported an association between virologic suppression on second line ART and adherence measured by pharmacy refill over the long term since second line ART initiation. Pharmacy refill as an adherence measure over shorter time periods is more pragmatic and implementable.[19] In our study short term “interval gap” refill performed similarly to long term “overall” refill on ROC analysis, and out-performed other methods of determining short term adherence using pharmacy refills. We explored the ability of
differing durations of pharmacy refill from three to twelve months to predict VF: “interval crude” and “interval average” performed better with longer durations, but the best performing method, “interval gap”, performed similarly over all of the interval durations assessed. Grossberg et al demonstrated that a 90 day period of pharmacy refill was associated with VL change,[19] but refill periods shorter than 60 days may overcall imperfect adherence leading to unnecessary clinical interventions.[17] We found that 80% adherence by pharmacy refill over 4 months appeared to be a threshold for predicting virologic suppression (figure 2). However, there were small numbers of patients in the lower adherence strata, which limited our ability to determine a threshold. Others have reported an increased risk of VF with adherence <80% in observational studies of patients on boosted PI regimens.[8,20] A threshold of 80% adherence measured by pharmacy refill in the previous 4 months could be used to identify patients needing enhanced adherence support and rationalise use of VL testing in resource-limited settings. Most patients on second line ART experiencing VF were able to achieve virologic suppression with intensified adherence support in a study at a clinic in South Africa.[21] VF on second line ART is likely a result of poor adherence rather than resistance as several studies have found a low proportion of major PI mutations in patients with VF on second line ART.[10–12] Unfortunately, as a result of high cost, the routine use of GART in patients with VF on second line will not be widely available in most low-middle income countries. Van Zyl et al [12] suggested an algorithm to select patients in VF on second line for GART using lopinavir plasma and hair therapeutic drug monitoring. However, these pharmacokinetic measures are costly (although less costly
than GART) and have extremely limited availability in resource-limited settings. By contrast, short term adherence measured by pharmacy refill can be easily implemented, especially in clinics with electronic dispensing, without incurring large additional costs. VL monitoring could also be limited in patients who are clinically well and demonstrate good adherence by short term pharmacy refill.

Our study has several limitations. First, the rate of VF on second line in the McCord clinic was lower than reported in a recent systematic review of second line treatment outcomes in resource limited settings, possibly due to a high physician/nurse to patient ratio and reliable antiretroviral drug supply.[2] Therefore the findings may not be generalisable to public sector ART clinics in other settings. Second, during the study period there were several changes to the drug regimens used in second line therapy including the use of zidovudine in the NRTI backbone instead of didanosine which had specific dosing instructions and heat-stable Alluvia® instead of Kaletra® as the boosted protease inhibitor. Consequently, the new second line ART regimen is easier to take which may have improved adherence in more recent calendar years. Third, we excluded patients with zero adherence and suppressed VL on the grounds that they must have been collecting ART at another clinic. However, we had no way of determining this and it is possible that other patients may also have collected ART at other clinics, which would weaken associations between adherence and the virologic outcomes we assessed. Fourth, we lacked power for some of the associations we assessed, notably the duration of second line ART and risk for VF, and the determination of an adherence threshold for virologic suppression.
In conclusion, short term pharmacy refill is an easily implementable adherence measure that can be used in ART clinics to identify patients at risk of VF on second line ART. Future studies need to be conducted in larger cohorts from clinics with a range of virologic outcomes in order to determine a threshold of adherence for predicting virologic response and to evaluate whether VL testing and GART could be limited to patients with better adherence.

Competing interests

The authors declare that they have no competing interests.

Authors’ contributions

RC drafted the manuscript and managed the data collection. RL participated in the study design and performed the statistical analysis. AS performed the descriptive analysis and assisted with data preparation for the statistical analysis. RM assisted with data collection and contributed significantly to the manuscript. HS assisted both with the setup of the study and managing the data collection. MA and PL collected the data and assisted with data preparation. GM supervised the study and was a major contributor to the study design and final manuscript.
Acknowledgments

The authors thank McCord Hospital and Sinikithemba for their willingness to make the data available for this study, and Kristy Nixon and Melisha Pertab for their assistance with the data collection. GM is supported in part by the National Research Foundation of South Africa (unique reference number 85810). The Grantholder acknowledges that opinions, findings and conclusions or recommendations expressed in any publication generated by the NRF supported research are that of the authors, and that the NRF accepts no liability whatsoever in this regard.
Figure legends

**Figure 1.** Area under the receiver operator characteristics curve comparing “interval gap”, “interval average” and “interval crude” short term pharmacy refill methods over varying durations prior to the viral load of interest. Error bars denote 95% confidence intervals.

**Figure 2.** Pharmacy refill adherence, measured by the “interval gap” method over 4 months, and virologic suppression. Error bars denote upper 95% confidence intervals.
REFERENCES


# TABLE 1

**Patient characteristics at initiation of second line ART**
*(numbers in parentheses = interquartile range).*

<p>| | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>No of females/males</td>
<td>149/125</td>
</tr>
<tr>
<td>Median age in years</td>
<td>35 (32 – 42)</td>
</tr>
<tr>
<td>Median CD4 at baseline (n=251)</td>
<td>174 (107 – 265)</td>
</tr>
<tr>
<td>Median log(_{10}) VL at baseline (n=261)</td>
<td>4.1 (3.6 – 4.7)</td>
</tr>
<tr>
<td>Median no of months followed up</td>
<td>27 (15 – 47)</td>
</tr>
</tbody>
</table>
TABLE 2
VL suppression over time. Note that the 6 monthly VL data reflect a window (e.g. a VL between 9 and 15 months was categorised as a 12 month VL).

<table>
<thead>
<tr>
<th>Months after starting second line</th>
<th>6</th>
<th>12</th>
<th>18</th>
<th>24</th>
<th>30</th>
<th>36</th>
<th>42</th>
<th>48</th>
</tr>
</thead>
<tbody>
<tr>
<td>No of patients in care</td>
<td>252</td>
<td>228</td>
<td>180</td>
<td>146</td>
<td>112</td>
<td>87</td>
<td>72</td>
<td>54</td>
</tr>
<tr>
<td>No with VL results</td>
<td>223</td>
<td>197</td>
<td>160</td>
<td>124</td>
<td>97</td>
<td>78</td>
<td>69</td>
<td>45</td>
</tr>
<tr>
<td>No with VL &lt;50</td>
<td>159</td>
<td>155</td>
<td>115</td>
<td>97</td>
<td>77</td>
<td>58</td>
<td>48</td>
<td>33</td>
</tr>
<tr>
<td>No with VL &lt;400</td>
<td>195</td>
<td>169</td>
<td>132</td>
<td>107</td>
<td>88</td>
<td>69</td>
<td>60</td>
<td>40</td>
</tr>
<tr>
<td>No with VL ≥1000</td>
<td>22</td>
<td>26</td>
<td>26</td>
<td>16</td>
<td>7</td>
<td>7</td>
<td>2</td>
<td>4</td>
</tr>
</tbody>
</table>

(Percentages in parentheses)
TABLE 3
Factors associated with VF among patients on second line ART

<table>
<thead>
<tr>
<th>Variables</th>
<th>Univariate</th>
<th>Multivariate</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Odds ratio (95% CI)</td>
<td>p-value</td>
</tr>
<tr>
<td>Adherence over 4 months (per 10% increase)</td>
<td>-0.46 (-0.66 to -0.26)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Time on second line ART</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>First year</td>
<td>referent</td>
</tr>
<tr>
<td></td>
<td>After first year</td>
<td>0.26 (-0.29 to 0.80)</td>
</tr>
<tr>
<td>Sex</td>
<td>Male</td>
<td>0.72 (-0.21 to 1.66)</td>
</tr>
<tr>
<td></td>
<td>Female</td>
<td>referent</td>
</tr>
<tr>
<td>Age (years)</td>
<td>&lt;26</td>
<td>1.7 (-2.16 to 5.57)</td>
</tr>
<tr>
<td></td>
<td>≥26</td>
<td>referent</td>
</tr>
<tr>
<td>Log$_{10}$ Viral load (copies/ml) at baseline</td>
<td>0.31 (-0.26 to 0.87)</td>
<td>0.286</td>
</tr>
<tr>
<td>Square-root CD4 (cells/μL) at baseline</td>
<td>-0.23 (-0.36 to -0.11)</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>
FIGURE 1

Area under the curve

Duration prior to VL (months)

- Blue circles: Interval gap
- Red squares: Interval average
- Green triangles: Interval crude
FIGURE 2

% Adherence 4 months prior to viral load

Proportion suppressed

VL <=50
VL <= 400

% Adherence 4 months prior to viral load

0.0  0.2  0.4  0.6  0.8  1.0
0 to 39  40 to 69  70 to 89  90 to 99  100

Proportion suppressed
PART C: ADDENDUM

FIGURE 3

Area under the receiver operator characteristics curve comparing two different methods of short term pharmacy refill, “interval gap” and “interval average” with long term “overall average” pharmacy refill methods over varying durations prior to the viral load of interest. Error bars denote 95% confidence intervals.
28 February 2012

HREC REF: 075/2012

Prof G Maartens
Division of Clinical Pharmacology
K-Floor
OMB

Dear Prof Maartens

PROJECT TITLE: ROLE OF ADHERENCE, ASSESSED BY PHARMACY REFILLS AND LOPINAVIR PLASMA CONCENTRATIONS, IN PROTEASE INHIBITOR FAILURE.

Thank you for submitting your study to the Faculty of Health Sciences Human Research Ethics Committee for review.

It is a pleasure to inform you that the Ethics Committee has formally approved the above-mentioned study.

Approval is granted for one year till the 28 February 2013.

Please submit a progress form, using the standardised Annual Report Form (FHS016), if the study continues beyond the approval period. Please submit a Standard Closure Form (FHS010) if the study is completed within the approval period.

Before the study may begin, please provide the HREC approval letter from Mc Hospital.

Please note that the ongoing ethical conduct of the study remains the responsibility of the principal investigator.

Please quote the REC. REF in all your correspondence.

Yours sincerely

[Signature]

PROFESSOR M BLOCKMAN
CHAIRPERSON, HSF HUMAN ETHICS

Federal Wide Assurance Number: FWA00001637.
Institutional Review Board (IRB) number: IRB000001938

[Signature]
19 March 2012

McCord Research Ethics Committee (MREC)
SA NHREC registration number: REC-170408-002
IRB00005803

Dear Dr Court

I refer to your application for ethics review of your amendment to the SARCS umbrella research study protocol. Your amendment to the umbrella study was reviewed by an expedited review of MREC. I have the pleasure in informing you that the amendment has now been approved.

TITLE OF PROJECT TO BE AMENDED: Molecular Characterization of HIV-1 Protease Cleavage Sites in South African Patients Failing HAART: viral fitness and drug resistance in HIV-1 subtype C

STUDY NUMBER OF PROJECT TO BE AMENDED: 310807/4.1 hs

NAME OF AMENDMENT STUDY: Role of Adherence, Assessed by Pharmacy Refills and Lopinavir Plasma Concentrations in Protease Inhibitor Failure

INVESTIGATOR (S): R Court (applicant)
G Maartens
H Sunpath

MREC DATE APPROVED: 19 March 2012

DECISION OF COMMITTEE: Full approval (expedited review)

Please note that any changes/amendments to this amendment must be reviewed and approved before being implemented. May we wish you every success in your research.

Sincerely

Dr Claire Kerry
Research Coordinator, McCord Hospital
**BioMed Central**

**Instructions for authors (abbreviated)**

**Overview of manuscript sections for Research articles**

Manuscripts for Research articles submitted to *BMC Infectious Diseases* should be divided into the following sections (in this order):

- Title page
- Abstract
- Keywords
- Background
- Methods
- Results and discussion
- Conclusions
- List of abbreviations used (if any)
- Competing interests
- Authors' contributions
- Authors' information
- Acknowledgements
- Endnotes
- References
- Illustrations and figures (if any)
- Tables and captions
- Preparing additional files

**Title page**

The title page should:

- provide the title of the article
- list the full names, institutional addresses and email addresses for all authors
- indicate the corresponding author

Please note:

- the title should include the study design, for example "A versus B in the treatment of C: a randomized controlled trial X is a risk factor for Y: a case control study"
- abbreviations within the title should be avoided

**Abstract**

The Abstract of the manuscript should not exceed 350 words and must be structured into separate sections: Background, the context and purpose of the study; Methods, how the study was performed and statistical tests used;
Results, the main findings; **Conclusions**, brief summary and potential implications. Please minimize the use of abbreviations and do not cite references in the abstract.

**Keywords**
Three to ten keywords representing the main content of the article.

**Background**
The Background section should be written in a way that is accessible to researchers without specialist knowledge in that area and must clearly state - and, if helpful, illustrate - the background to the research and its aims. Reports of clinical research should, where appropriate, include a summary of a search of the literature to indicate why this study was necessary and what it aimed to contribute to the field. The section should end with a brief statement of what is being reported in the article.

**Methods**
The methods section should include the design of the study, the setting, the type of participants or materials involved, a clear description of all interventions and comparisons, and the type of analysis used, including a power calculation if appropriate. Generic drug names should generally be used. When proprietary brands are used in research, include the brand names in parentheses in the Methods section.

**Results and discussion**
The Results and discussion may be combined into a single section or presented separately. Results of statistical analysis should include, where appropriate, relative and absolute risks or risk reductions, and confidence intervals. The Results and discussion sections may also be broken into subsections with short, informative headings.

**Conclusions**
This should state clearly the main conclusions of the research and give a clear explanation of their importance and relevance. Summary illustrations may be included.

**List of abbreviations**
If abbreviations are used in the text they should be defined in the text at first use, and a list of abbreviations can be provided, which should precede the competing interests and authors' contributions.
Competing interests
A competing interest exists when your interpretation of data or presentation of information may be influenced by your personal or financial relationship with other people or organizations. Authors must disclose any financial competing interests; they should also reveal any non-financial competing interests that may cause them embarrassment were they to become public after the publication of the manuscript.
Authors are required to complete a declaration of competing interests. All competing interests that are declared will be listed at the end of published articles. Where an author gives no competing interests, the listing will read 'The author(s) declare that they have no competing interests'.

Authors’ contributions
In order to give appropriate credit to each author of a paper, the individual contributions of authors to the manuscript should be specified in this section. According to ICMJE guidelines, An 'author' is generally considered to be someone who has made substantive intellectual contributions to a published study. To qualify as an author one should 1) have made substantial contributions to conception and design, or acquisition of data, or analysis and interpretation of data; 2) have been involved in drafting the manuscript or revising it critically for important intellectual content; 3) have given final approval of the version to be published; and 4) agree to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.
Each author should have participated sufficiently in the work to take public responsibility for appropriate portions of the content. Acquisition of funding, collection of data, or general supervision of the research group, alone, does not justify authorship.
We suggest the following kind of format (please use initials to refer to each author’s contribution): AB carried out the molecular genetic studies, participated in the sequence alignment and drafted the manuscript. JY carried out the immunoassays. MT participated in the sequence alignment. ES participated in the design of the study and performed the statistical analysis. FG conceived of the study, and participated in its design and
coordination and helped to draft the manuscript. All authors read and approved the final manuscript.

All contributors who do not meet the criteria for authorship should be listed in an acknowledgements section. Examples of those who might be acknowledged include a person who provided purely technical help, writing assistance, or a department chair who provided only general support.

**Authors’ information**

You may choose to use this section to include any relevant information about the author(s) that may aid the reader's interpretation of the article, and understand the standpoint of the author(s). This may include details about the authors' qualifications, current positions they hold at institutions or societies, or any other relevant background information. Please refer to authors using their initials. Note this section should not be used to describe any competing interests.

**Acknowledgements**

Please acknowledge anyone who contributed towards the article by making substantial contributions to conception, design, acquisition of data, or analysis and interpretation of data, or who was involved in drafting the manuscript or revising it critically for important intellectual content, but who does not meet the criteria for authorship. Please also include the source(s) of funding for each author, and for the manuscript preparation. Authors must describe the role of the funding body, if any, in design, in the collection, analysis, and interpretation of data; in the writing of the manuscript; and in the decision to submit the manuscript for publication. Please also acknowledge anyone who contributed materials essential for the study. If a language editor has made significant revision of the manuscript, we recommend that you acknowledge the editor by name, where possible.

The role of a scientific (medical) writer must be included in the acknowledgements section, including their source(s) of funding. We suggest wording such as 'We thank Jane Doe who provided medical writing services on behalf of XYZ Pharmaceuticals Ltd.'

Authors should obtain permission to acknowledge from all those mentioned in the Acknowledgements section.
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Endnotes should be designated within the text using a superscript lowercase letter and all notes (along with their corresponding letter) should be included in the Endnotes section. Please format this section in a paragraph rather than a list.

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All references, including URLs, must be numbered consecutively, in square brackets, in the order in which they are cited in the text, followed by any in tables or legends. Each reference must have an individual reference number. Please avoid excessive referencing. If automatic numbering systems are used, the reference numbers must be finalized and the bibliography must be fully formatted before submission.

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Any in press articles cited within the references and necessary for the reviewers' assessment of the manuscript should be made available if requested by the editorial office.

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- Reference Manager
- Zotero

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All web links and URLs, including links to the authors’ own websites, should be given a reference number and included in the reference list rather than within the text of the manuscript. They should be provided in full, including both the title of the site and the URL, in the following format: The Mouse Tumor Biology Database [http://tumor.informatics.jax.org/mtbwi/index.do]. If an author or group of authors can clearly be associated with a web link, such as for weblogs, then they should be included in the reference.

Preparing illustrations and figures

Illustrations should be provided as separate files, not embedded in the text file. Each figure should include a single illustration and should fit on a single page in portrait format. If a figure consists of separate parts, it is important that a single composite illustration file be submitted which contains all parts of the figure. There is no charge for the use of color figures.

Please read our figure preparation guidelines for detailed instructions on maximising the quality of your figures.

Formats

The following file formats can be accepted:

- PDF (preferred format for diagrams)
- DOCX/DOC (single page only)
- PPTX/PPT (single slide only)
- EPS
- PNG (preferred format for photos or images)
- TIFF
- JPEG
- BMP

Figure legends

The legends should be included in the main manuscript text file at the end of the document, rather than being a part of the figure file. For each figure, the following information should be provided: Figure number (in sequence, using Arabic numerals - i.e. Figure 1, 2, 3 etc); short title of figure (maximum 15 words); detailed legend, up to 300 words.

Please note that it is the responsibility of the author(s) to obtain permission from the copyright holder to reproduce figures or tables that have previously been published elsewhere.
Preparing tables

Each table should be numbered and cited in sequence using Arabic numerals (i.e. Table 1, 2, 3 etc.). Tables should also have a title (above the table) that summarizes the whole table; it should be no longer than 15 words. Detailed legends may then follow, but they should be concise. Tables should always be cited in text in consecutive numerical order.

Smaller tables considered to be integral to the manuscript can be pasted into the end of the document text file, in A4 portrait or landscape format. These will be typeset and displayed in the final published form of the article. Such tables should be formatted using the 'Table object' in a word processing program to ensure that columns of data are kept aligned when the file is sent electronically for review; this will not always be the case if columns are generated by simply using tabs to separate text. Columns and rows of data should be made visibly distinct by ensuring that the borders of each cell display as black lines. Commas should not be used to indicate numerical values. Color and shading may not be used; parts of the table can be highlighted using symbols or bold text, the meaning of which should be explained in a table legend. Tables should not be embedded as figures or spreadsheet files.

Larger datasets or tables too wide for a portrait page can be uploaded separately as additional files. Additional files will not be displayed in the final, laid-out PDF of the article, but a link will be provided to the files as supplied by the author.

Tabular data provided as additional files can be uploaded as an Excel spreadsheet (.xls) or comma separated values (.csv). As with all files, please use the standard file extensions.

Style and language

General

Currently, *BMC Infectious Diseases* can only accept manuscripts written in English. Spelling should be US English or British English, but not a mixture. There is no explicit limit on the length of articles submitted, but authors are encouraged to be concise.

*BMC Infectious Diseases* will not edit submitted manuscripts for style or language; reviewers may advise rejection of a manuscript if it is
compromised by grammatical errors. Authors are advised to write clearly and simply, and to have their article checked by colleagues before submission. In-house copyediting will be minimal. Non-native speakers of English may choose to make use of a copyediting service.

**Abbreviations**

Abbreviations should be used as sparingly as possible. They should be defined when first used and a list of abbreviations can be provided following the main manuscript text.

**Typography**

- Please use double line spacing.
- Type the text unjustified, without hyphenating words at line breaks.
- Use hard returns only to end headings and paragraphs, not to rearrange lines.
- Capitalize only the first word, and proper nouns, in the title.
- All lines and pages should be numbered. Authors are asked to ensure that line numbering is included in the main text file of their manuscript at the time of submission to facilitate peer-review. Once a manuscript has been accepted, line numbering should be removed from the manuscript before publication. For authors submitting their manuscript in Microsoft Word please do not insert page breaks in your manuscript to ensure page numbering is consistent between your text file and the PDF generated from your submission and used in the review process.
- Use the *BMC Infectious Diseases* reference format.
- Footnotes are not allowed, but endnotes are permitted.
- Please do not format the text in multiple columns.