

## Sensitive Tenofovir Resistance Screening of HIV-1 From the Genital and Blood Compartments of Women With Breakthrough Infections in the CAPRISA 004 Tenofovir Gel Trial

Xierong Wei,<sup>1</sup> Gillian Hunt,<sup>4</sup> Salim S. Abdool Karim,<sup>2,6</sup> Vivek Naranbhai,<sup>6</sup> Sengeziwe Sibeko,<sup>6</sup> Quarraisha Abdool Karim,<sup>2,6</sup> Jin-fen Li,<sup>1</sup> Angela D. M. Kashuba,<sup>3</sup> Lise Werner,<sup>6</sup> Jo-Ann S. Passmore,<sup>5,6</sup> Lynn Morris,<sup>4</sup> Walid Heneine,<sup>1</sup> and Jeffrey A. Johnson<sup>1</sup>

<sup>1</sup>Centers for Disease Control and Prevention, Atlanta, Georgia; <sup>2</sup>Department of Epidemiology, Columbia University, New York, New York; <sup>3</sup>Eshelman School of Pharmacy, University of North Carolina, Chapel Hill; <sup>4</sup>National Institute for Communicable Diseases and <sup>5</sup>National Health Laboratory Service, Johannesburg, and <sup>6</sup>CAPRISA—Centre for the AIDS Programme of Research in South Africa, University of KwaZulu-Natal, Durban

**The Centre for the AIDS Programme of Research in South Africa 004 (CAPRISA 004) study demonstrated that vaginally applied tenofovir gel is a promising intervention for protecting women from sexually acquiring human immunodeficiency virus (HIV). However, the potential for emergence of tenofovir resistance remains a concern in women who seroconvert while using the gel despite the lack of plasma virus resistance as assessed by population sequencing during the trial. We applied highly sensitive polymerase chain reaction–based assays to screen for tenofovir resistance in plasma and vaginal swab specimens. The absence of mutation detection suggested little immediate risk of tenofovir-resistant HIV-1 emergence and forward transmission in settings in which gel users are closely monitored for HIV seroconversion.**

**Keywords.** Vaginal microbicide; HIV prevention; pre-exposure prophylaxis; tenofovir gel; topical PrEP.

Globally, women are disproportionately affected by human immunodeficiency virus (HIV) infection and the availability of efficacious female-controlled products for preventing virus

acquisition is essential to alleviating this burden. In the wake of several trials of barrier microbicides that demonstrated no protection against HIV, vaginal microbicides containing antiretroviral drugs have become leading candidates for the prevention of HIV sexual transmission in women. The Centre for the AIDS Programme of Research in South Africa 004 trial (CAPRISA 004) assessed the efficacy of 1% tenofovir gel in sexually active women and identified a 39% lower HIV incidence in the tenofovir gel arm compared with the placebo arm, an effective incidence rate ratio of 0.61 (confidence interval, .4–.94;  $P = .02$ ) [1]. Subsequent examination of vaginal aspirates with quantifiable tenofovir concentrations found that seroconversion in the tenofovir gel arm was associated with a lower tenofovir concentration geometric mean (634.85 ng/mL) than in women who remained protected (7582.76 ng/mL;  $P = .09$  [ $t$  test]).

As with any intervention involving antiretroviral drugs, emergence of drug resistance is a concern. Trace concentrations of tenofovir can be detected in vaginal aspirates up to 30 days after gel application; thus, the long suboptimal tenofovir tail may provide an environment for transmitted virus to develop drug resistance. In the primary CAPRISA 004 data analysis [1], bulk sequence genotyping of tenofovir gel breakthrough infections identified no drug resistance in plasma virus several months after seroconversion. However, there were limitations to this resistance analysis. Foremost, the time since seroconversion and, hence, drug exposure was approximately 5 months, which may have been sufficiently long to allow for resistance to decay below detection by population genotyping. Moreover, topically applied drug remains concentrated in the vaginal compartment with little systemic exposure. Therefore, drug pressure may not have been sufficient for resistance mutations to emerge or become detectable in the peripheral circulation when examined by bulk sequencing.

In this study, we applied polymerase chain reaction (PCR)–based mutation screening assays with improved sensitivity to reexamine HIV seroconverters in the CAPRISA 004 trial for evidence of tenofovir drug resistance in plasma virus that might have emerged at levels below what could be detected by bulk sequence analysis. Moreover, we evaluated whether virus colocalized with tenofovir gel in the vaginal compartment might result in higher levels of the K65R resistance mutation than what was observed for plasma. The combination of assays with improved sensitivity for mutation detection, analysis of samples collected closer to the time of gel use and seroconversion, and examination of HIV in the vaginal compartment afforded a more rigorous assessment of both drug resistance transmission and emergence in this tenofovir vaginal gel trial.

Received 23 August 2013; accepted 29 November 2013; electronically published 16 January 2014.

Correspondence: Jeffrey A. Johnson, PhD, 1600 Clifton Rd NE, MS G45, Atlanta, GA 30333 (jjohnson1@cdc.gov).

**The Journal of Infectious Diseases** 2014;209:1916–20

Published by Oxford University Press on behalf of the Infectious Diseases Society of America 2014.

This work is written by (a) US Government employee(s) and is in the public domain in the US.

DOI: 10.1093/infdis/jiu026

## MATERIALS AND METHODS

### CAPRISA 004 Population and Sampling

The CAPRISA 004 study (ClinicalTrials.gov identifier NCT00441298) enrolled 899 sexually active women from urban and rural sites, who were aged 18–40 years and were not pregnant, and randomly assigned them equally to a 1% tenofovir hydroxyethylcellulose gel arm or a hydroxyethylcellulose placebo gel arm. Subtype C HIV seroconversion occurred in 38 of 422 (9%) women in the tenofovir gel arm and in 60 of 421 (14%) in the placebo arm.

In the current study, we evaluated all available plasma and vaginal swab samples obtained closest to the estimated time of seroconversion. For plasma virus, PCR amplicons from 65 seroconverters, 28 (74%) from the tenofovir arm and 37 (62%) from the placebo arm, were screened by means of sensitive resistance testing at the Centers for Disease Control and Prevention. The samples were masked as to the gel arms at the time of testing. Seventy-five percent of the plasma specimens were collected within 30 days of estimated seroconversion (median, 14 days). Although all earliest available samples were examined, the priority for testing were women who reported applying gel within 30 days before the estimated date of seroconversion. The University of KwaZulu-Natal's Biomedical Research Ethics committee approved the study, and the institutional review board of the Centers for Disease Control and Prevention determined that participant consent was provided for the HIV testing in this study.

Because vaginal lavage samples were unavailable for genital HIV analysis, we evaluated cervicovaginal swab samples (ProbeTec; Becton-Dickinson) collected at the first scheduled study visit after infection, when available, and stored in 0.5 mL of phosphate-buffered saline (PBS) at  $-80^{\circ}\text{C}$ . A total of 33 swab samples from the tenofovir arm and 28 from the placebo arm were tested. Before we elected to use the study swab samples for HIV genotyping, we devised an extraction protocol and assessed nucleic acid recovery using plasma samples of known viral loads (see [Supplementary Information](#)).

### Vaginal Virus Loads and Drug Concentrations

Vaginal viral loads were measured at the National Health Laboratory Service Diagnostic Laboratory at Groote Schuur Hospital in South Africa, using a 1-mL fraction of 10 mL of PBS cervicovaginal lavage in the Nuclisens Easyq HIV 1 kit, version 1.2 (detection limit, 50 copies/mL). Tenofovir concentrations were quantified in 50  $\mu\text{L}$  of directly aspirated cervicovaginal fluid; the drug was extracted by using an acetonitrile protein precipitation process (see [Supplementary Information](#)). We defined the minimum appreciable drug concentration as 10 ng/mL.

### HIV Nucleic Acid Amplification and Drug Resistance Testing

For real-time PCR drug resistance screening, virus template from blood plasma was first generated as described elsewhere

[2]. For drug resistance screening from vaginal swab samples, nucleic acids from the pooled PBS fractions (see [Supplementary Information](#)) were extracted using the UltraSens viral RNA kit (Qiagen) and eluted in a final 80- $\mu\text{L}$  volume of buffer provided with the kit. Five microliters of the extract was used in reverse-transcription PCR amplification of the viral template, as described elsewhere [3], except that High Fidelity PCR (Life Technologies) was used in the current study.

The PCR-generated viral templates from blood and swab samples were screened for the K65R (reaction is positive if the difference in total virus copy versus mutation copy amplification ( $\Delta\text{CT}$ ) was  $\leq 8$  cycles) and K70E (positive  $\Delta\text{CT}$ ,  $\leq 7$  cycles) tenofovir resistance mutations using real-time PCR-based (allele-specific) point mutation assays validated for subtype C HIV, as described elsewhere ([4] and [Supplementary Information](#)). Mutation-specific assay reactions that screened positive were directly sequenced to verify that reading frames were intact and mutation-positive underwent clonal analysis to verify mutation frequency. In addition, the original reverse-transcription PCR products were bulk genotyped to ascertain whether mutations were at sufficiently high frequency to be identifiable by population sequencing.

## RESULTS

### Plasma Drug Resistance and Tenofovir Exposure

Sensitive real-time PCR screening of 65 HIV seroconverter plasma masked for gel arm found that only one participant, participant 16, was positive for the K65R mutation ( $\Delta\text{CT}$ , 3.7) in a sample collected 3 weeks after infection. The remaining 64 specimens had no K65R reactivity within the cutoff. After unmasking, it was disclosed the K65R-positive specimen was from a participant assigned to the placebo gel arm (Tables 1 and 2; [Supplementary Table 3](#)). A subsequent bulk sequence analysis revealed that she also had K103N and Y181C mutations to nonnucleoside drugs not related to tenofovir. The remainder of the placebo plasma samples had  $\Delta\text{CT}$  values of  $\geq 8.4$  cycles (median, 9.9 cycles), which were similar to the  $\geq 8.1$  cycles (median, 9.8 cycles) obtained for the tenofovir arm plasma samples.

### Vaginal Tenofovir Concentrations

Tenofovir concentrations in cervicovaginal aspirates collected around the time of swab sampling were available for 27 seroconverters from the tenofovir arm. Seven aspirates (26%) had drug concentrations  $> 2$  ng/mL (Table 2), ranging from  $< 0.25$  to 213 000 ng/mL (geometric mean, 4.12 ng/mL). Furthermore, analysis of available preseroconversion aspirates from the remaining 11 women who experienced seroconversion in the tenofovir arm revealed only 3 women had detectable tenofovir in any sample.

**Table 1. Testing Summary for CAPRISA 004 Seroconverters**

Testing Status or Outcome	Tenofovir Gel (n = 38)	Placebo Gel (n = 60)
Plasma tested, No. (%)	28 (74)	37 (62)
Swab samples tested, No. (%)	33 (87)	28 (47)
Swab samples amplifiable, No. (%)	21 (55)	21 (35)
Estimated duration of infection at swab sample collection, median (range), d	19 (3–156)	19 (12–157)
Used gel within last 30 d, %	45	43
Interval since application, range, d	0–20	0–16
Applicators used in last 30 days, median (mean), No.	2 (3.8)	0 (2.9)
Vaginal tenofovir >2 ng/mL, No. (%)	7/27 (26)	...
Resistance detected below cutoff, No. (%)		
K65R (plasma)	0	1 (2.7)
K65R (swab samples)	0	0
K70E (plasma and swab samples)	0	0

Abbreviation: CAPRISA 004, Centre for the AIDS Programme of Research in South Africa 004.

### Swab Sample Analysis

For the tenofovir resistance analysis of cervicovaginal swab samples, we selected the earliest available postseroconversion samples, thus the samples obtained closest to the time of anticipated last gel use. Thirty-three swab samples from the tenofovir arm (87% of seroconverters) and 28 from the placebo arm swab (47% of seroconverters) were available for testing. The estimated durations of infection at the times of swab sample collection ranged from 3 to 156 days (median, 19 days) for the tenofovir arm and from 12 to 157 days (median, 19 days) for the placebo arm seroconverters. Paired plasma and swab samples were available for 38 participants, of which 23 were from the tenofovir and 15 from the placebo arm. Testing the tenofovir arm swab samples with the K65R assay identified no samples with reactivity below the cutoff ( $\Delta$ CT  $\geq$ 9.7 cycles; median, 12.8 cycles) (Table 2). These results were similar to those obtained in testing the placebo arm swab samples ( $\Delta$ CT,  $\geq$ 9.1 cycles; median, 13.1 cycles; [Supplementary Table 3](#)). No K70E was identified from any swab specimens, which had  $\Delta$ CT values of  $\geq$ 9.7 cycles for tenofovir arm and  $\geq$ 10.0 cycles for placebo arm seroconverters.

### DISCUSSION

In this sensitive screening study for HIV drug resistance in vaginal swab and plasma samples from seroconverting women who participated in CAPRISA 004, we found no evidence of tenofovir resistance associated with having been assigned tenofovir vaginal gel. The cryopreserved swab specimens proved useful for examining expression of tenofovir resistance in the genital

compartment where the topically applied tenofovir is primarily concentrated. The tenofovir arm swab specimens produced similar results to the placebo gel arm in the sensitive testing, providing no indication of potential early resistance emergence with tenofovir gel and no evidence of drug-resistant virus as a cause of breakthrough infection.

Apart from the encouraging finding that the tenofovir gel afforded significant protection when used, 9% of women assigned to tenofovir gel did experience seroconversion, and for a few, HIV-1 RNA was detected in the vaginal compartment even when tenofovir was present at appreciable concentrations. However, 74% of the seroconverters tested in the tenofovir arm had insignificant vaginal drug concentrations, even with self-reported gel use, revealing that adherence to gel use was very low in these women and is likely to explain the majority of seroconversions in the tenofovir gel group.

From the plasma and vaginal samples collected soon after infection, we found the prevalence of transmitted resistance in the study was low, with only 1 participant, a woman assigned to the placebo arm, having evidence of infection with a drug-resistant variant. The absence of tenofovir-associated mutations in the tenofovir gel arm supports the conclusion that none of those women became infected as a result of virus that was resistant to tenofovir. The uncommon occurrence of transmitted K65R is consistent with findings from surveillance of newly diagnosed [5] and other evaluations of antiretroviral drug-naïve infections [6]. This bodes well for biomedical interventions that currently use tenofovir; however, systematic monitoring for transmission of this mutation would be timely as use of tenofovir in HIV treatment expands. Oral prophylaxis trials [7–9] have also examined drug resistance and, as with CAPRISA 004, have identified no evidence of drug resistance emergence in persons who seroconverted during the study period.

This drug resistance study had a few important limitations; foremost, the apparent poor adherence to gel application in the tenofovir seroconverters, as evidenced by the paucity of detectable tenofovir in the vaginal aspirates, would have made emergence of resistance unlikely. Another limitation to studying drug resistance risk in this trial setting was the frequent monitoring of participants and immediate removal of study gel when seroconversion was confirmed, thereby possibly not allowing sufficient time for tenofovir resistance to emerge. Microbicide implementation evaluations that involve less-frequent monitoring could be better positioned to assess the potential for resistance emergence with longer exposures to tenofovir in cases of HIV acquisition. Nonetheless, some insight into the likelihood of drug resistance emergence can be gleaned from nonhuman primate studies. In 1 study, macaques exposed to repeated tenofovir gel applications for 10 weeks after simian-human immunodeficiency virus breakthrough infections provided no evidence of K65R when examined with sensitive assays [10], suggesting that seroconversion while using tenofovir gel is not likely to pose an immediate risk of drug resistance.

**Table 2. Sensitive Resistance Testing of Plasma and Swab Samples From Tenofovir Arm Seroconverters<sup>a</sup>**

Participant	Time After Infection, d	Gel <30d <sup>b</sup>	Plasma VL, Copies/mL <sup>c</sup>	Plasma ΔCT, Cycles		CVL VL, Copies/mL	VA Tenofovir Concentration, ng/mL	Swab Sample ΔCT, Cycles	
				K65R <sup>d</sup>	K70E <sup>e</sup>			K65R <sup>d</sup>	K70E <sup>e</sup>
1 <sup>f</sup>	37	Yes	22 500	9.0	12.6	...	...	...	...
	99	No	16 500	...	...	...	<0.25	12.8	>25
2 <sup>f</sup>	21	Yes	1000	11.2	12.9	...	...	...	...
	121	No	<500	...	...	...	<0.25	NA	NA
3	21	Yes	6500	12.3	13.1	<50	6	18.2	>25
4	271	No	1500	9.1	12.7	...	<0.25	...	...
10	13	No	1000	22.0	12.5	<50	28	NA	NA
13	96	No	1500	11.6	14.1	...	...	NA	NA
14	14	Yes	6000	9.5	11.7	...	<0.25	14.0	9.7
15 <sup>f</sup>	34	Yes	10 000	9.8	11.4	...	...	...	...
	-29 <sup>g</sup>	Yes	10 000	...	...	...	78	NA	NA
18	15	Yes	13 000	9.3	11.5	...	745	...	...
20 <sup>f</sup>	23	Yes	22 000	9.4	>25	...	...	...	...
	114	No	7000	...	...	...	...	NA	NA
23	14	Yes	109 000	12.3	9.4	51 000	206	13.4	19.9
26	35	No	199 500	9.6	10.7	100	<0.25	NA	NA
27	14	Yes	179 500	13.1	11.4	...	<0.25	15.1	>25
30	58	Yes	4 433 000	8.5	10.8	...	<0.25	NA <sup>h</sup>	10.8
31	14	Yes	88 500	9.6	14.4	...	<0.25	11.7	>25
32 <sup>f</sup>	21	Yes	15 000	8.1	11.8	...	...	...	...
	115	No	26 500	...	...	...	<0.25	NA	NA
34	20	Yes	52 500	8.9	10.4	...	...	11.2	>25
35	13	Yes	220 500	10.4	10.0	700	213 103	10.1	>25
36	14	Yes	258 000	11.3	11.5	300	<0.25	11.5	14.0
40	141	No	305 000	11.7	11.7	...	<0.25	11.6	15.7
42	15	Yes	534 000	9.9	20.3	2000	<0.25	14.3	>25
44	14	No	246 000	8.2	12.2	54 000	<0.25	9.7	>25
45 <sup>f</sup>	52	No	130 000	13.0	>25	...	...	...	...
	3	No	330 000	...	...	...	6119	NA	NA
46	18	Yes	124 000	8.1	10.0	...	18 707	...	...
50	14	Yes	37 000	9.2	9.9	55 000	<0.25	>25	10.9
52	15	Yes	18 000	8.7	21.4	1800	<0.25	11.7	>25
55	14	Yes	500	10.1	12.0	...	...	...	...
65	48	No	216 500	12.2	>25	...	...	15.7	>25
66	121	No	30 000	...	...	...	<0.25	>25	>25
67	14	Yes	48 500	...	...	...	12	NA	NA
68	48	No	31 000	...	...	<50	<0.25	NA	NA
69	14	Yes	500	...	...	<50	<0.25	NA	NA
70	156	No	17 500	...	...	...	<0.25	11.6	>25
71	135	No	111 000	...	...	400	...	NA	NA
72	19	No	1 700 000	...	...	7800	<0.25	11.5	>25
73	154	No	298 000	...	...	...	<0.25	13.0	>25
74	123	No	<500	...	...	...	...	NA	NA

Abbreviations: ΔCT, difference in total virus copy amplification cycle and mutation-specific amplification cycle; CVL, cervicovaginal lavage; NA, not amplifiable; VA, vaginal aspirate.

<sup>a</sup> Empty cells (. . .) indicate data unavailable.

<sup>b</sup> Gel used in past 30 days.

<sup>c</sup> Rounded to nearest 500 copies.

<sup>d</sup> Cutoff, 8.0 cycles.

<sup>e</sup> Cutoff, 7.0 cycle.

<sup>f</sup> Matched blood and swab samples were sampled from different days.

<sup>g</sup> Tested for evidence of early acute infection before seroconversion; participant seroconverted later, but no subsequent swab specimens were available.

<sup>h</sup> Insufficient template.

In the current study, sensitive testing of both plasma and vaginal swab specimens produced no remarkable findings of tenofovir-induced drug resistance. However, a possible long-term impact on subsequent tenofovir-containing antiretroviral treatment regimens for women who seroconverted in the tenofovir gel arm is being assessed in the CAPRISA 009 trial. In conclusion, the findings from this study demonstrated no adverse drug resistance outcome from the use of tenofovir vaginal gel and do not raise concerns over continuing clinical evaluations of this female-controlled product as an intervention against sexual acquisition of HIV.

## Supplementary Data

Supplementary materials are available at *The Journal of Infectious Diseases* online (<http://jid.oxfordjournals.org/>). Supplementary materials consist of data provided by the author that are published to benefit the reader. The posted materials are not copyedited. The contents of all supplementary data are the sole responsibility of the authors. Questions or messages regarding errors should be addressed to the author.

## Notes

**Acknowledgments.** The findings and conclusions expressed in this article are those of the authors and do not necessarily represent the official view of the Centers for Disease Control and Prevention. The views expressed by the authors do not necessarily reflect those of United States Agency for International Development (USAID), Gilead Sciences, Eastern Virginia Medical School, or Contraception Research and Development (CONRAD).

**Financial support.** This work was supported by Centers for Disease Control and Prevention intramural funding. The parent trial (CAPRISA 004) was supported by USAID, FHI360 (USAID cooperative agreement GPO-A-00-05-00022-00, contract 132119), and the Technology Innovation Agency (LIFElab) of the South African government's Department of Science and Technology. Tenofovir was provided by Gilead Sciences, and the gel was manufactured and supplied for the CAPRISA 004 trial by CONRAD. The current studies are part of the CAPRISA TRAPS (Tenofovir Gel Research for AIDS Prevention Science) Program, which is funded by CONRAD, Eastern Virginia Medical School (USAID cooperative grant GP00-08-00005-00, subproject agreement PPA-09-046). The University of North Carolina at Chapel Hill Center for AIDS Research is funded by the National Institutes of Health (grant P30 AI50410).

**Potential conflicts of interest.** Jeffrey A. Johnson and Walid Heine are inventors on a patent filed (PCT US2012/025638) for the mutation-specific PCR assays. Salim Abdool Karim and Quarraisha Abdool Karim were co-principal investigators for the CASPRISA 04 trial and are coinventors for 2 pending patents (61/354.050 and 61/357.892) for tenofovir gel against herpes simplex virus types 1 and 2 with scientists from Gilead Sciences. All other authors report no potential conflicts.

All authors have submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest. Conflicts that the editors consider relevant to the content of the manuscript have been disclosed.

## References

1. Abdool Karim Q, Abdool Karim SS, Frohlich JA, et al. Effectiveness and safety of tenofovir gel, an antiretroviral microbicide, for the prevention of HIV infection in women. *Science* **2010**; 329:1168–74.
2. Loubser S, Balfe P, Sherman G, Hammer S, Kuhn L, Morris L. Decay of K103N mutants in cellular DNA and plasma RNA after single-dose nevirapine to reduce mother-to-child HIV transmission. *AIDS* **2006**; 20:995–1002.
3. Johnson JA, Li J-F, Wei X, et al. Simple PCR assays improve the sensitivity of HIV-1 subtype B drug resistance testing and allow linking of resistance mutations. *PLoS ONE* **2007**; 2:e638.
4. Li JF, Lipscomb JT, Wei X, et al. Detection of low-level K65R variants in nucleoside reverse transcriptase inhibitor-naive chronic and acute HIV-1 subtype C infections. *J Infect Dis* **2011**; 203:798–802.
5. Wheeler WH, Ziebell RA, Zabina H, et al. Prevalence of transmitted drug resistance associated mutations and HIV-1 subtypes in new HIV-1 diagnoses, U.S.-2006. *AIDS* **2010**; 24:1203–12.
6. Chan PA, Huang A, Kantor R. Low prevalence of transmitted K65R and other tenofovir resistance mutations across different HIV-1 subtypes: implications for pre-exposure prophylaxis. *J Int AIDS Soc* **2012**; 15:17701–6.
7. Grant RM, Lama JR, Anderson PL, et al. Preexposure chemoprophylaxis for HIV prevention in men who have sex with men. *N Engl J Med* **2010**; 363:2587–99.
8. Baeten JM, Donnell D, Ndase P, et al. Antiretroviral prophylaxis for HIV prevention in heterosexual men and women. *N Engl J Med* **2012**; 367:399–410.
9. Thigpen MC, Kebaabetswe PM, Paxton LA, et al. Antiretroviral pre-exposure prophylaxis for heterosexual HIV transmission in Botswana. *N Engl J Med* **2012**; 367:423–34.
10. Dobard C, Sharma S, Martin A, et al. Durable protection from vaginal simian-human immunodeficiency virus infection in macaques by tenofovir gel and its relationship to drug levels in tissue. *J Virol* **2012**; 86:718–25.