Clinica Chimica Acta 560 (2024) 119723

Contents lists available at ScienceDirect

Clinica Chimica Acta

journal homepage: www.elsevier.com/locate/cca

Stable hemoglobin concentration with fecal immunochemical test at high temperatures in a Caribbean colorectal cancer screening program

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ARTICLE INFO

Keywords:
Fecal immunochemical test
Hemoglobin concentration
Colorectal cancer
Temperature
Colorectal cancer screening

ABSTRACT

Background and aims: High temperatures may reduce fecal immunochemical test (FIT) positivity and colorectal cancer (CRC) detection sensitivity. We investigated the effect of temperature on hemoglobin concentration [Hb], in the FOB Gold®. Additionally, we examined FIT pick-up, storage, return times and specimen collection.
Materials and Methods: In vitro experiments with buffer containing FIT devices, inoculated with Hb-spiked stool. For 7 days, 144 samples were stored in groups of 36 at 4 °C, 22 °C, 30 °C, and 50 °C. Additionally, 54 samples were stored in groups of 18 at 34 °C, 42 °C and 50 °C for 20 h. Paired t-tests and repeated measure ANOVA assessed [Hb] change. Sixty-five screening participants completed a FIT-handling questionnaire.
Results: After 7 days, mean [Hb] was stable at 30 °C (0.8 μg Hb/g;95 %CI: −1.5 to 3.1;$p = 0.50$). For 50 °C, mean [Hb] decreased within 2 days (−21.3 μg Hb/g;95 %CI: −30.2 to −12.5;$p < 0.001$) and after 20 h (−63.0 μg Hb/g;95 %CI: −88.7 to −37.3;$p < 0.001$), respectively. All other temperature categories showed significant mean [Hb] increase. Same-day FIT return was reported by 80 %. Eighty-seven percent experienced specimen collection as easy and 33 % kept the FIT refrigerated after collection.
Conclusions: The FOB Gold® is suitable for CRC screening in tropical climates. Although most respondents indicated same-day sample return, we recommend avoiding FIT storage above 30 °C for longer than7 days.

1. Introduction

Colorectal cancer (CRC) screening has been shown to be a cost-effective measure to reduce CRC incidence and CRC-related mortality over time by way of early detection [1–3]. Fecal Immunochemical Testing (FIT) is used in population-based CRC screening programs to identify individuals at risk for CRC and its precursor lesions. As one of the first countries in the Caribbean, Curaçao launched its own population-based screening program for CRC in 2020 using the FOB Gold®. Participants who test positive are referred for colonoscopy, while those with a negative result are re-invited after two years [4].

The FIT detects human hemoglobin (Hb) through antibodies. Unlike its predecessor, the guiac fecal occult blood test, FIT requires no preparation or dietary restrictions before testing and is completed with one sample [5–8]. Pooled sensitivity and specificity for CRC detection are 74 % and 94 % respectively, varying by the selected positivity threshold and population characteristics [9]. The threshold used in the Curaçao program is ≥ 8micrograms Hb per gram feces (μg Hb/g), which is lower than in most other CRC screening programs [10].

The climate in Curaçao is tropical, average temperatures range from 27 to 30 °C throughout the year [11]. In European countries, FIT-positivity rates have been shown to be relatively lower during summer months, implying that high ambient temperatures accelerate the breakdown of Hb in FIT devices [12–14]. Aside from ambient

Abbreviations: FIT, Fecal immunochemical test; Hb, Hemoglobin; CRC, Colorectal cancer.

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https://doi.org/10.1016/j.cca.2024.119723
Received 7 May 2024; Accepted 7 May 2024
Available online 10 May 2024

temperature, return times may also affect FIT performance. Long return times of FIT samples may increase the risk of false negative test results, because of time-related Hb degradation [15].

The evidence about the seasonal effects on Hb concentration suggests that the risk of a false negative FIT result may be higher on Curaçao than in regions with milder climates. However, data on the performance of the FOB Gold® in warmer climates is scarce.

This study evaluated Hb concentration in the FOB Gold® at a range of temperatures over time. Secondly, we aimed to gain insight into FIT pick-up, storage, return times and specimen collection method by invitees on the island.

2. Materials and methods

2.1. Setting

The Caribbean Prevention Center – Fundashon Prevenshon (CPC-FP) invites Curaçao residents, aged 50–75 years by postal mail to participate in the CRC screening program. Invitees can pick-up their FIT at one of the screening affiliated laboratories, FP main-office, selected pharmacies and a bus that visits neighbourhoods across the island offering the FIT and information. At distribution, practical instructions are given on how to collect the specimen at home, where to return the FIT, and how to receive the results. The standard recommendation is to return the FIT as soon as possible after specimen collection. If almost immediate return is not possible, then screenees are advised to store the sample in the refrigerator.

2.2. Study design

We conducted an in vitro technical validity study and a cross-sectional survey.

2.2.1. In vitro study

Two experiments were performed at Analytic Diagnostic Center (ADC) laboratory, Willemstad, Curaçao. We assessed the effects of temperature on Hb concentration during 7 days and 20 h. The ADC analyses FITs for the CRC screening program. The FIT has been set-up as a user-defined test on the Atellica Solution (*Siemens-Healthineers, Erlangen, Germany*) with FOB Gold® (*Sentinel Diagnostics, Milano, Italy*) reagents. The Hb concentration is turbidimetrically assessed on this automated chemistry analyzer. Latex particles coated with anti-human Hb bind to sample Hb.

2.2.2. Donor feces and sample preparation

Volunteers for feces donation were recruited from the FP main-office and social media page. All nine donors signed written informed consent before the experiments. Donations were stored at −20 °C after collection. Samples were thawed for two hours or until pliable before each experiment. ADC provided whole blood products.

We aimed to create FIT samples within a broad range of Hb concentrations, to increase the validity of the results. To meet this aim, we titrated blood to create low, medium and high concentration groups. First, we created the high concentration group with feces to blood ratios of 1.0 g to 1.5 (µl). Then to create the medium and low concentrations, we diluted the mixture using phosphate-buffered saline or additional fecal matter to achieve feces to blood ratios of 1.0 g to 1.0 µl, and 0.8 µl, respectively. After preparing the mixtures, FOB Gold® tubes were inoculated and allowed settle for 1 h before baseline analysis.

2.2.3. Seven (7) days of incubation

For the 7-day experiment, mixtures from the low, medium and high concentration groups were evenly divided into 144 FIT-tubes. Twelve tubes from each concentration group were then randomly subdivided into the temperature categories: 4 °C, 22 °C, 30 °C and 50 °C. Samples in the 4 °C group were stored in a refrigerator. Those in the 22 °C group were kept on a bench in an air-conditioned room. A water bath was used to store the samples at 30 °C (*Thermo Scientific Precision, Waltham, Massachusetts*) and those in the 50 °C group were stored in an oven (*Heratherm Thermo Scientific, Waltham, Massachusetts*).

The Hb concentrations were determined at baseline, 1 day, 2 days, 4 days and 7 days. All 144 tubes were tested at baseline and at day 7. To prevent inaccuracies due to possible limited buffer, half of all tubes from each temperature category were tested on day 1 and day 4, and the other half on day 2.

2.2.4. Twenty (20) hours of incubation

In total, 54 FIT tubes were prepared for the 20-hour experiment. The samples that were kept at 34 °C and 42 °C were prepared and analysed on the same-day. Due to limited available incubators, a separate batch of samples were prepared and kept at 50 °C. For each temperature group (34 °C, 42 °C and 50 °C) 18 samples were prepared and randomly subdivided into a low, medium and high concentration group resulting in six samples per category. After we learned from the previous experiment that there was enough buffer to test five times, the Hb concentrations for all samples were assessed at baseline, 2.5 h, 5 h, 10 h and 20 h.

2.3. Questionnaire study population

CRC screening participants who returned their FIT to the CPC-FP main office were approached to fill-in the FIT-handling questionnaire from May to October 2022. Informed consent was obtained from each respondent prior to questionnaire completion.

2.3.1. Questionnaire development

The research team, in collaboration with the CPC-FP developed the questionnaire. Before distribution, the questionnaire was pre-tested and a focus group was hosted to ensure validity. Open questions assessed sample return times and time between FIT pick-up and specimen collection by the CRC screening participants. We also included multiple-choice questions about specimen collection ease and practices. Lastly, questions about FIT storage before and after specimen collection were included (see Appendix A).

2.4. Statistical analysis

Hb concentrations (µg Hb/g) were assessed at baseline and then re-measured over time. The mean Hb concentration per temperature category was examined and compared using a paired *t*-test and repeated-measures ANOVA. P-values of 0.05 or lower were considered statistically significant.

The questionnaires were analyzed per question. Questions reflecting time intervals are presented in days. Multiple-choice questions are presented in percentages of respondents who choose each question option. R statistical programming was used for all analyses.

3. Results

3.1. Hb concentrations within 7 days of incubation

Fig. 1 illustrates the experiment set-up. The mean Hb concentration at baseline for all 144 FIT tubes was 22.0 µg Hb/g (SD 17.1) and 24.0 µg Hb/g (SD 27.7) on day 7. A repeated measure ANOVA did not show a significant interaction between time and temperature ($F = 0.466$, $p = 0.71$).

When data was stratified by temperature group, mean Hb concentration did not change in samples stored at 30 °C (0.8 µg Hb/g; 95 %CI: −1.5 to 3.1, $p = 0.50$). An increase in mean Hb concentration was observed in the 4 °C (13.6 µg Hb/g; 95 %CI: 8.4 to 18.7; $p < 0.001$) and 22 °C (11.9 µg Hb/g; 95 %CI: 6.7 to 17.0; $p < 0.001$) groups. We observed a significant decrease in mean Hb concentration from baseline

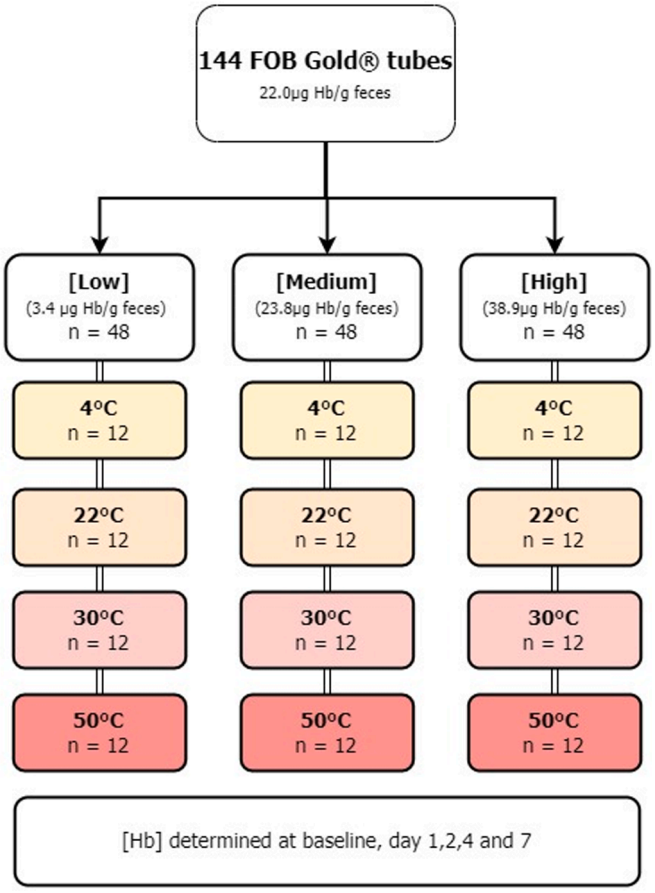

Fig. 1. Experiment set-up for 7 days of incubation. A total of 144 samples divided in low, medium, high concentration groups (n = 48) were stored in groups of 12 at: 4 °C (refrigerator), 22 °C (air-conditioned room), 30 °C (water bath) and 50 °C (incubator). Half of all samples were tested at baseline, day 1, day 4 and day 7. The other half was tested at baseline, day 2 and day 7.

to day 7 in samples stored at 50 °C (−18.5 µg Hb/g; 95 %CI: –23.8 to −13.2, $p < 0.001$). (Fig. 2).

3.2. Hb concentrations after 20 h incubation

For the temperature groups 34 °C and 42 °C, the mean Hb concentration at baseline was 15.1 µg Hb/g (SD 9.3). For the samples that were prepared separately and kept at 50 °C, the baseline mean was 84.6 µg Hb/g (SD 54.8) (Fig. 3).

We found an increase in mean Hb concentration between baseline and 2.5 h for the samples kept 34 °C (25.8 µg Hb/g; 95 % CI 19.4 to 32.2; $p < 0.001$) and 42 °C (19.7 µg Hb/g; 95 % CI 11.7 to 27.7; $p < 0.001$) (Fig. 4). Between baseline and 20 h, mean Hb concentration significantly increased at 34 °C (29.9 µg Hb/g; 95 % CI 22.4 to 37.5$p < 0.001$) and 42 °C (28.4 µg Hb/g; 95 % CI 18.6 to 38.1; $p < 0.001$). However, the repeated measure ANOVA for samples kept at 34 °C and 42 °C did not show a significant interaction between time and temperature (F = 0.912, p = 0.459). For the samples kept at 50 °C mean Hb concentration decreased significantly between baseline and 20 h by 63.0 µg Hb/g (95 % CI; −88.7 to −37.3; $p < 0.001$) (Fig. 4 and Table 1).

3.3. Questionnaire

In total, 65 questionnaires were returned to the CPC-FP with only 3 % missing data across all variables (Table 2). Sex was recorded for 56 questionnaires (77 % women). The median age of the respondents was 64 years.

The completed FIT was returned on the same-day by 80 % of the respondents; almost half (49 %) of the respondents reported doing so within one hour after sample collection. Before sample collection, 55.5 % of respondents reported keeping the FIT in a non-air-conditioned room. After sample collection, only 33 % reported keeping the sample in the refrigerator as advised in the brochure. Sample collection was experienced as easy (7–10) by 87 % of the respondents. See Table 2.

4. Discussion

In this study, we assessed the effect of temperature and time on Hb concentration in the FOB Gold®. We also used a questionnaire to gain insight into FIT pick-up, storage, return times and specimen collection amongst CRC screening participants in Curaçao. Our data shows that mean Hb concentration of the FIT does not decrease for 7 days for temperatures between 4–30 °C. However, Hb concentration clearly showed a rapid decrease when stored at 50 °C. The majority of questionnaire respondents indicated same-day sample return, experienced specimen collection as easy, and did not refrigerate the FIT before or after specimen collection. Our findings suggest that screening participants using the FOB Gold® need an additional warning not to leave the FIT samples in places where temperatures exceed those of the ambient environment, such as direct sunlight.

The Hb concentration stability we detected at 30 °C is in line with Sentinel Diagnostics' reports that Hb degradation is limited for up to 14 days at 30 °C after stool sampling [16]. Symonds *et al.* stored 59 OC-Sensor (Eiken Chemical Co., Tokyo, Japan) samples from CRC screening participants in Australia at 35 °C. Similar to our results, they described an 80 % retention of the baseline Hb concentration for up to 8 days [17]. In contrast to our results, Dancourt *et al.* studied test stability of the FOB Gold® in 2012. They found a significant decrease in Hb concentration after 4 days, when samples were stored at 30 °C [18]. Their study was conducted with positive samples from the regular CRC screening program, which may explain the differences compared to our observations.

The most prominent change in mean Hb concentration was observed at 50 °C. All samples in the 7 day experiment measured below the limit of detection at the first follow-up point, albeit that baseline Hb concentrations were already relatively low. In the 20 h experiment, where baseline concentrations were higher, the mean Hb concentration decreased by 63 µg Hb/g (75 %, $p < 0.001$) within 20 h. Symonds *et al.* stored 56 OC-Sensor (Eiken Chemical Co., Tokyo, Japan) samples in a dry oven at 50 °C. Similar to our findings, they reported a significant decrease below 80 % of the original Hb concentration within the first 2 days [17].

We observed an increase in mean Hb concentration for samples stored at 4 °C, 22 °C (7 days of incubation), 34 °C and 42 °C (20 h of incubation). During the 20-hour experiment, the increase occurred within the first 2.5 h after which the mean Hb concentration stabilized. This may indicate that it takes time for Hb to properly saturate in the buffer and form complexes [19]. This however does not hold for 4 °C and 22 °C, where the increase is after 1–2 days. We could speculate that Hb settling is somewhat temperature related. Our results also suggest that the point at which Hb rapidly degrades lies somewhere between 42 °C and 50 °C. This is further supported by the manufacturer's claim that up to 87 % of original Hb concentration can be recovered from samples kept at 36–38 °C for 7 days [16].

Results from other studies for temperatures similar to 4 °C and 22 °C are mixed. A 2005 Israeli study found that the Hb degradation was not significant for a period of 21 days for samples stored at 20 °C [20]. Catomeris *et al.* conducted a study with 33 tubes from five different FIT devices (not including the FOB Gold®) stored at four different temperatures, including the refrigerator (4–8 °C) and room temperature (20–22 °C). They found decreasing recoveries with increasing temperature and time. They also indicated that refrigeration offered the best stability [21]. However, in our study there was little to no difference in

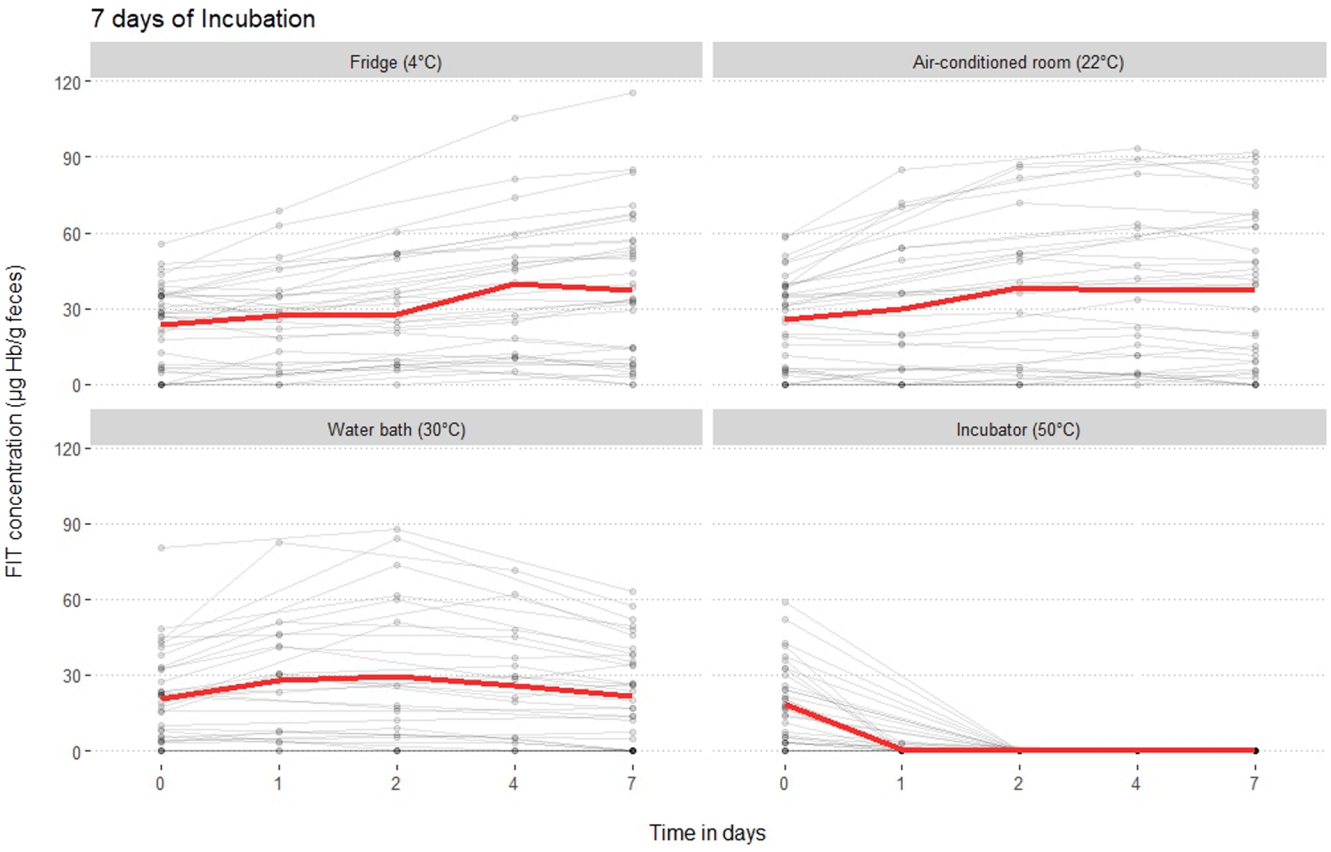

Fig. 2. Mean Hb concentration (y-axis) during 7 days of incubation (x-axis), measured for 4 °C, 22 °C, 30 °C and 50 °C (n = 36 per temperature group).

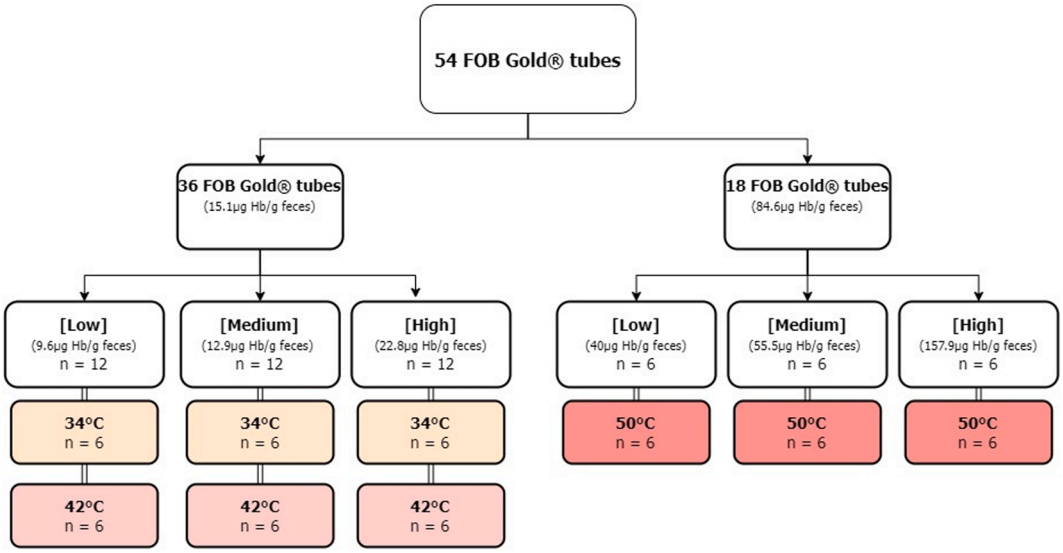

Fig. 3. Experiment set-up for 20 h incubation. Thirty-six samples divided in low, medium, high concentration groups (n = 12) stored in groups of six at 34 °C (water incubator) and 42 °C (incubator). Later, 18 samples were stored at 50 °C (incubator), with six samples per concentration category.

mean Hb concentration change between 4 °C and 22 °C. In a German study, Gies *et al.*, the FOB Gold® was compared to other FIT devices. Although most other devices showed stable Hb concentrations for 7 days when kept at 5 °C, the FOB Gold® showed a significant decrease in Hb concentration. The same was observed for tubes kept at 20 °C [22]. The differences in results of the afore-mentioned in vitro studies may by caused by differences in concentration ranges, FIT devices, sample origin and preparation, number of times a sample was analysed, sample sizes and observation time-intervals [23]. Furthermore, the 7-day time-cap may have hindered the observation of possible overall stability.

Delays in sample return have also been reported as a possible cause for false negative results [15]. Fortunately, the questionnaires show that almost all respondents returned samples within 2 days, with the vast majority even doing so on the same-day. This is largely in line with previous findings, 81 % of a population of 3767 described by van Rossum *et al.* returned the FIT within 4 days [15]. It is also sooner than the

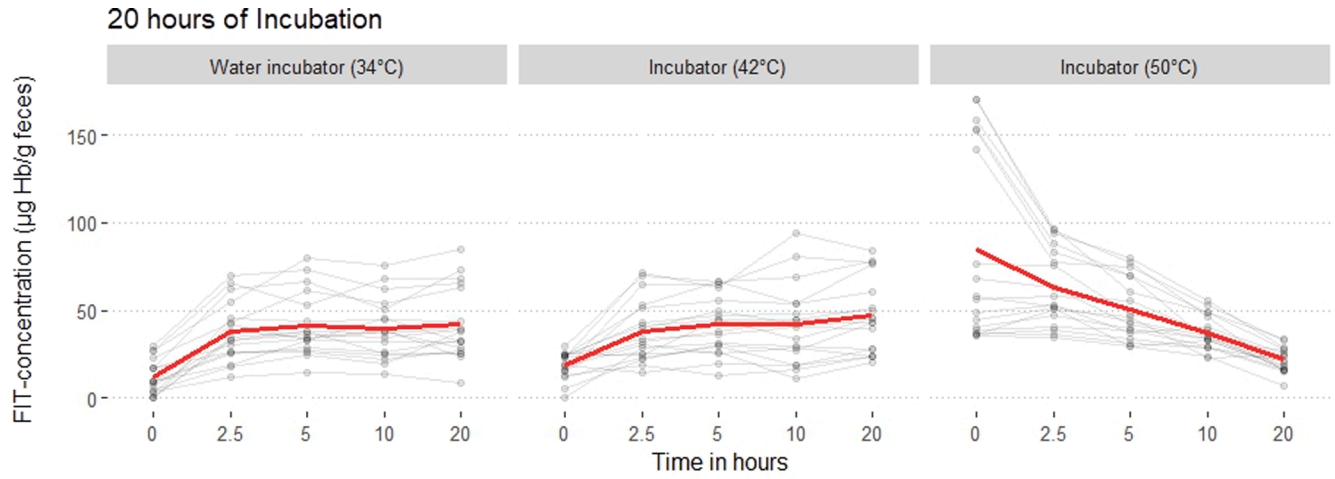

Fig. 4. Mean Hb concentration (y-axis) during 20 h of incubation (x-axis), measured for 34 °C, 42 °C and 50 °C (n = 18 per temperature group).

Table 1
Hb concentrations, 7 days and 20 hours of incubation.

7 days of Incubation				
Temperature group	**Number of samples (n)**	**Baseline Mean (SD)**	**Day 7 Mean (SD)**	***P*-Value**
4 °C	36	23.6 (16.2)	37.2 (28.5)	*< 0.001*
22 – 24 °C	36	25.4 (18.3)	37.3 (31.2)	*< 0.001*
30 °C	36	20.6 (18.1)	21.4 (19.5)	0.50
50 °C	36	18.5 (15.7)	0 (0)	*< 0.001*
20 h of Incubation				
Temperature group	**Number of samples (n)**	**Baseline Mean (SD)**	**20 Hour Mean (SD)**	***P*-Value**
34 °C	18	11.8 (9.9)	41.7 (20.6)	< 0.001
42 °C	18	18.4 (7.5)	46.8 (21)	< 0.001
50 °C	18	84.6 (54.8)	21.6 (7.0)	< 0.001

Mean Hb concentration (µg Hb/g), SD and P values per temperature group at baseline and last measurement moment for 7 days and 20 h of incubation.

Table 2
Results of FIT-handling questionnaire (n = 65).

Question	**n (%)**	**n**
Storage before sample collection		63
Air-conditioned room	10 (16)	
Non air-conditioned room	35 (55.5)	
Refrigerator	12 (19)	
Somewhere else	6 (9.5)	
Storage after sample collection		64
Air-conditioned room	6 (9)	
Non air-conditioned room	27 (42)	
Refrigerator	21 (33)	
Brought it back immediately	9 (14)	
Somewhere else	1 (2)	
Storage during transportation		61
Ambient	47 (77)	
Chilled	14 (23)	
Time between pick-up and sample collection		63
Same-day	20 (32)	
Next day	14 (22)	
2 days – 1 week	19 (30)	
1 – 2 weeks	6 (10)	
> 2 weeks	4 (6)	
Time between sample collection and return		65
Same-day	52 (80)	
Next day	12 (18)	
After 2 days	1 (2)	
Time to test return		63
Within 1 h	31 (49)	
1–2 h	21 (33)	
2–4 h	5 (8)	
> 4 h	6 (10)	
Excrement made contact with water during sample collection		64
Yes	5 (8)	
No	59 (92)	
Material used during sample collection		65
Nothing	2 (3)	
Toilet paper	10 (15)	
Other type of paper	7 (11)	
Cardboard	19 (29)	
Foil	1 (2)	
Other	26 (40)	
Sample collection difficulty		62
Very easy (9–10)	36 (58)	
Easy (7–8)	18 (29)	
Relatively easy – neutral (5–6)	7 (11)	
Difficult (<5)	1 (2)	
Assistance received during sample collection		63
None	60 (95)	
Yes, from family member or friend	1 (2)	
Yes, from someone else	2 (3)	

mean sample return of approximately 4 days reported by Dancourt *et al* [18]. Van Rossum *et al.* also reported a significant decrease in positivity rates for tests returned ≥ 7 days [15]. In contrast, van Roon *et al.* did not find a decrease in positivity or detection rate for return times up to 10 days [24]. Similarly, in a Danish study where 60 OC-Sensor samples were kept at 30 °C and re-tested after 14 days no positive tests became negative [25]. The short return time reported by current respondents in combination with the low cut-off, limits concerns for possible false negatives. At 50 °C, the temperature with the most pronounced mean Hb concentration decrease, only one positive test became negative using the Curaçao cut-off, 8 µg Hb/g. If the American threshold (20 µg Hb/g) were to be adopted, then an additional seven samples would have become negative.

Considering Curaçao's ambient temperature, the storage conditions after sample collection are a matter of concern. The majority of our respondents did not report keeping the FIT refrigerated before or after sample collection. With the quick return and observed stability at 30 °C in mind, we recommend advising immediate return and avoiding direct sunlight.

A strength of the current study is the controlled laboratory setting. This allowed us to look at the effects of specific temperatures over time on the FOB Gold®. The robustness of our sample size allows us to draw strong conclusions from our observations. Furthermore, the use of the FOB Gold® specifically adds to the available literature. When it comes to in vitro studies focussing on the FIT, the OC sensor (Eiken Chemical Co.,

Tokyo, Japan) has been analysed most often [15,17,24,25].

However, there are also some limitations. Most of these encompass the sample preparation before tube inoculation. Firstly, we collected feces from healthy volunteers. We did not test the donations for blood prior to mixing, so whether or not the composition of the fecal matter itself had an effect on the results is unknown. However, feces were randomly mixed among the four temperature categories. Secondly, because we conducted the experiments on three separate occasions, there are differences in baseline Hb concentrations. Therefore, feces and blood related inter-batch differences cannot be ruled out. We prepared all of the samples ourselves and mixed the feces and blood together by hand. Consequently, despite our best efforts, complete homogenization is not guaranteed. All parts of the experiment were standardized: we used fixed feces to blood ratios and followed the manufacturer's testing protocol during analysis. Lastly, the set upper limit of the measuring was 170 μg Hb/g, which may have influenced the absolute and relative change in Hb concentration across temperature and time. However, it is unlikely that this influenced the direction of the change i. Furthermore, the current range of Hb concentration was realistic and included most cut-off points used in screening programs. Arguably, changes in Hb around the cut-off point are of most clinical value, since these will lead to changes in the number of positives.

As for the questionnaires, the insight the current results offer into how the CRC screening population on Curaçao handles the FIT is a strength. This was a previously unexplored area. Furthermore, we requested that respondents fill-in the questionnaire immediately after return. This limits possible recall bias. Nevertheless, there are also limitations. Only 65 respondents completed the questionnaire and all respondents were recruited at the CPC-FP main office. In the interest of a representative sample and limitation of error, more questionnaires from as many FIT drop-off locations as possible would have been preferred. Volunteer bias may have also played a role. The possibility exists that those who were inclined to do so are individuals who often do what is expected of them and thus we cannot rule out that socially desired answers were given.

5. Conclusions

The FOB Gold® is suitable for use in tropical climates, considering the average ambient temperature range is 27–30 °C and Hb concentration was stable for up to 7 days at 30 °C. Most questionnaire respondents indicated same-day FIT return. We recommend that screenees avoid sample storage above 30 °C, in addition to the current advice to return samples as soon as possible to the laboratory.

CRediT authorship contribution statement

Shacara N. Blake: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Project administration, Resources, Writing – original draft. **Tim L. Kortlever:** Conceptualization, Data curation, Formal analysis, Methodology, Visualization, Writing – review & editing. **Sue Ellen Verbrugge:** Data curation, Methodology, Resources, Writing – review & editing. **Anneke J. van Vuuren:** Conceptualization, Methodology, Writing – review & editing. **Evelien Dekker:** Conceptualization, Supervision, Writing – review & editing. **Manon van der Vlugt:** Conceptualization, Methodology, Supervision, Writing – review & editing. **Jacqueline G. Hugtenburg:** Conceptualization, Methodology, Supervision, Writing – review & editing.

Data availability

Data will be made available on request.

Acknowledgements

The authors would like to thank Leonard Makaai for his help with sample analysis. We would also like to express our gratitude to Nina Kooij for her assistance in getting the necessary materials. A special thanks to the ADC for facilitating the experiments. Lastly, the authors would like to express gratitude to the CPC-FP for facilitating questionnaire collection.

Funding

This work was supported by the Stichting Preventie Fonds (SPF).

Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.cca.2024.119723.

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