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Evaluation of the impact of pre-analytical conditions on sample stability for the detection of SARS-CoV-2 RNA

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ABSTRACT

Demand for accurate SARS-CoV-2 diagnostics is high. Most samples in the UK are collected in the community and rely on the postal service for delivery to the laboratories. The current recommendation remains that swabs should be collected in Viral Transport Media (VTM) and transported with a cold chain to the laboratory for RNA extraction and RT-qPCR. This is not always possible. We aimed to test the stability of SARS-CoV-2 RNA subjected to different pre-analytical conditions. Swabs were dipped into PBS containing cultured SARS-CoV-2 and placed in either a dry tube or a tube containing either normal saline or VTM. The tubes were then stored at different temperatures (20–50 °C) for variable periods (8 h to 5 days). Samples were tested by RT-qPCR targeting SARS-CoV-2 E gene. VTM outperformed swabs in saline and dry swabs in all conditions. Samples in VTM were stable, independent of a cold chain, for 5 days, with a maximum increase in cycle threshold (Ct) of 1.34 when held at 40 °C. Using normal saline as the transport media resulted in a loss of sensitivity (increased Ct) over time and with increasing temperature (up to 7.8 cycles compared to VTM). SARS-CoV-2 was not detected in 3/9 samples in normal saline when tested after 120 h incubation. Transportation of samples in VTM provides a high level of confidence in the results despite the potential for considerable, uncontrolled variation in temperature and longer transportation periods. False negative results may be seen after 96 h in saline and viral loads will appear lower.

1. Introduction

The reliance on SARS-CoV-2 diagnostic testing by real-time, reverse transcriptase polymerase chain reaction (RT-PCR) to track and contain the virus spread shows no sign of slowing. The process continues to evolve as the assays become more robust, but fundamental to all diagnostics is sample collection. It therefore remains pertinent to improve our understanding of how different pre-analytical conditions, such as sampling and transportation methods, may impact results and thus the implementation of measures to control the pandemic.

Ideally, the testing process involves a correctly taken nasopharyngeal swab stored in viral transport medium (VTM) at 2–4 °C for no more than 24 h before processing. The use of VTM and a cold chain is the current recommendation by the WHO (World Health Organization, 2020). However, this is not always affordable nor achievable, and there are concerns about potential toxicity if these are sent unsupervised for home testing. Currently samples from community testing in the UK (whether they be postal, home tests or in satellite testing centres) are transported at ambient temperature until they reach the laboratory at which point, they are stored at 2–4 °C until processing. Furthermore, if a circumstance arises in which a sample needs to be re-tested these are potentially kept at room temperature for several hours. Previously there has been a shortage of viral transport medium (VTM) due to the sudden increase in demand for laboratory supplies. Should issues like this recur it is useful to know how different media may affect the detection of SARS-CoV-2 RNA. It is also not always possible to maintain a cold chain, therefore knowing the impact of different storage temperatures is of importance. Previous studies have demonstrated the stability of other media types for swab storage including normal saline (0.9 % NaCl) and Phosphate-Buffered Saline (PBS) but with a cold chain or freezing of samples (Rodino et al., 2020). In other studies, a high-titre viral sample has been used and consequently differences in sensitivity would not necessarily impact the diagnosis (Rogers et al., 2020). Dry samples in a carefully regulated cold chain have been used successfully in the

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REACT-1 study but this is expensive requiring couriers (Riley et al., 2021).

The aim of this study was to investigate the impact of different pre-analytical variables on the recovery of SARS-CoV-2 RNA. Swabs were stored in VTM, saline and in no media (dry) at different temperatures for varying incubation times to replicate the sampling and transportation conditions and SARS-CoV-2 recovery quantified.

2. Materials and methods

2.1. Study design

Specimen Collection Swabs (NP Swab II, NEST, Wuxi NEST Biotechnology Co. Ltd) were used for the dry swabs and Specimen Collection Swab with Flocked Tip and Molded Breakpoint (Trafalgar Scientific) for the swabs in VTM and in saline. The swabs were placed in PBS containing cultured SARS-CoV-2 virus at a concentration of ~23,7000 copies/ml (delta strain (B.1.617.2), for 5 s during which time the swab absorbs ~ 100uL of the supernatant, equivalent to approximately 23,700 copies. Initial virus concentration is determined by real-time RT- PCR and the copy number extrapolated from standard curve of a dilution series of a synthetic RNA standard as previously described (Rowan et al., 2021). Swabs were then placed in a dry tube (NEST disposable sampler, Wuxi NEST Biotechnology Co. Ltd); in normal saline (NEST disposable sampler 2.0 ml, Wuxi NEST Biotechnology Co. Ltd) or in Viral Transport Medium/VTM (Σ-VIROCULT®, MWE, Lot 21C13) Sigma Virocult® is a balanced salt solution, buffered with disodium hydrogen orthophosphate, and contains lactalbumin hydrolysate as a stabiliser, and antibiotics (Chloramphenicol and Amphotericin) to inhibit the growth of any bacterial contaminants in the specimen. All conditions were tested in triplicate. All samples were first chilled at 2–8 °C for 1 h. For the first stage of this investigation, each swab was then heated to one of three temperatures: room temperature (~20 °C); 30 °C or 50 °C and incubated for either 8 h or 24 h (to mimic potential ambient temperatures during transportation without a cold chain). Each swab was then stored at 2–8 °C for 60 h to mimic potential refrigeration at the destination laboratory prior to RNA extraction (Method summarised in Fig. 1A). All samples were extracted in a single run. To replicate the recommended cold chain the three swab types were also tested (in triplicate) following storage of the swabs at 2–8 °C for 22 h as a control).

As the second part of this investigation, the two media types that had yielded the best results were tested with longer storage times at the various temperatures (up to 120 h/5 days). Swabs were prepared as previously described and kept at 2–8 °C for 24 h to mimic refrigeration at home pending arrival of a courier or taking the sample to a postal collection point. Swabs were then kept at room temperature (~20 °C), 30 °C or 40 °C for 24 h, 48 h, 96 h or 120 h. After this, all samples were kept at 2–8 °C, for 24–48 h prior to RNA extraction in a single run. (Method summarised in Fig. 1B).

2.2. RNA extraction and RT-PCR

For all samples, viral RNA was extracted using an automatic platform (CyBio FeliX liquid handler) with the innuPREP Virus DNA/RNA Kit (Analytik Jena) as previously described (Crone et al., 2020). For the dry swabs, 1 ml of 60 % Lysis buffer (diluted with phosphate-buffered saline without calcium chloride or magnesium chloride) was added and left for 10 min before the aliquot of lysis buffer for extraction was processed (Moore et al., 2008). SARS-CoV-2 RNA (Viral E gene) was detected by real-time RT- PCR (Rowan et al., 2021) (Fig. 1).

3. Results

The recovery of SARS-CoV-2 RNA from dry swabs, and swabs stored with VTM or normal saline, for up to 24 h, was determined. The mean Ct, standard deviation and variance for each condition are displayed in Table 1. Fig. 2A and B show an increase in Ct value of each swab type at each temperature at 8hrs and 24hrs respectively, compared to a swab of same type prepared in the same way but stored at 2–8 °C for 22hrs before processing, to mimic the cold chain conditions.

The Ct values of the samples in VTM were stable across all temperature conditions and times with a maximum increase in Ct of 0.4 (Table 1). For normal saline swabs, a loss in sensitivity with increase in temperatures and incubation times was observed, with an increase in Ct of 2.6 when stored at 50 °C for 24 h. With the dry swabs there was an average increase in Ct of 2.7 cycles (maximum 3 cycles) representing an 8-fold loss of RNA. Viral RNA was not recoverable from one of the dry

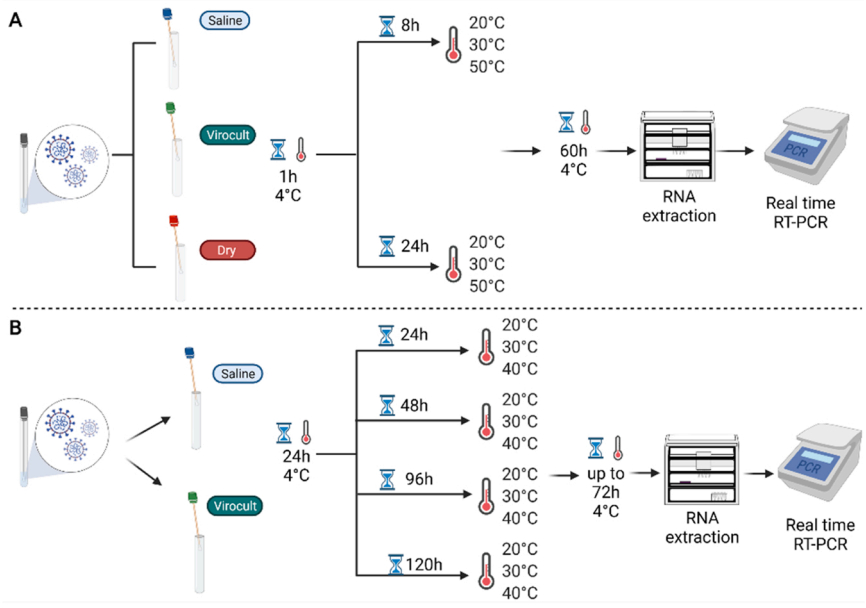

Fig. 1. A schematic overview of the methods used in this study from swab set-up and variable pre-analytical conditions to RT-qPCR A. Methods used in the first part of this study which included the use of dry swabs and only went to 24 h incubation. B. Methods for the second part which used Saline and VTM swabs with incubation up to 120 h/5 days. Image created with biorender.com.

Table 1
Displays the mean Ct values (of the three replicates) for each condition tested, alongside standard deviation, % CV (Coefficient of Variation), and Ct difference Ct difference is compared to the same sample type refrigerated for 22 h.

Condition	Mean Ct	St Dev	%CV	Ct Difference (vs. 22 h at 4 °C)
8 h				
20 °C				
VTM	30.56	0.23	0.75	0.42
Saline	32.78	0.53	1.62	0.58
Dry	35.69	1.49	4.17	2.09
30 °C				
VTM	29.78	0.26	0.87	-0.36
Saline	32.79	1.22	3.72	0.59
Dry	35.93	2.95	8.21	2.33
50 °C				
VTM	30.15	0.11	0.36	0.01
Saline	33.8	2.24	6.63	1.6
Dry	36.68	1	2.73	3.08
24 h				
20 °C				
VTM	30.01	0.17	0.57	-0.13
Saline	34.33	0.69	2.01	2.13
Dry	36.29	1.48	4.08	2.69
30 °C				
VTM	29.97	0.5	1.67	-0.17
Saline	33.88	2.27	6.70	1.68
Dry	36.55	1.06	2.90	2.95
50 °C				
VTM	29.68	0.34	1.15	-0.46
Saline	34.86	1.31	3.76	2.66
Dry*	36.68	0.59	1.61	3.08

* Sample not detected

swabs kept at 50 °C for 24 h (Fig. 2A).

The results of incubation of swabs for up to 120 h, are presented in Fig. 3 with the mean Ct, standard deviation and variance for each condition shown in Table 2. As observed over 24 h, virus in VTM generated stable Ct values across the experimental conditions with a range of mean Ct from 28.42 to 29.76 (standard deviation 0.46). In contrast, there was increasing loss of virus in normal saline with a progressive increase in Ct with temperature and time. The mean Ct values increased by 4.57 from 31.73 to 36.3 (which equates to >20-fold reduction in viral RNA compared to baseline). Thus, compared to VTM the increase in Ct values for normal saline, ranges from 2.09 for virus stored for 24 h at 20 C to 7.84 when stored for 120 h at 40 °C. This loss of viral RNA is seen with both increase in temperature and duration of storage, with a greater impact resulting from storage at temperatures higher than 20 °C (Fig. 3). SARS-CoV-2 RNA was detected in all samples kept in VTM, however 1/3 and 2/3 replicates stored in normal saline for 120 h at 30 °C and 40 °C respectively had no detectable RNA.

4. Discussion

Existing studies on the stability of SARS-CoV-2 RNA have mainly focused on different pre-analytical conditions such as time and temperature using VTM and normal saline or PBS as the swab media. The use of dry swabs for SARS-CoV-2 is an inexpensive and safe alternative to swabs in transport media and good concordance of qualitative results for up to 48 h in the absence of a cold chain has been reported (Padgett et al., 2021), (Padgett et al., 2021), despite an up to 3-cycle increase in Ct. Others have shown an average 2-cycle increase with dry swabs

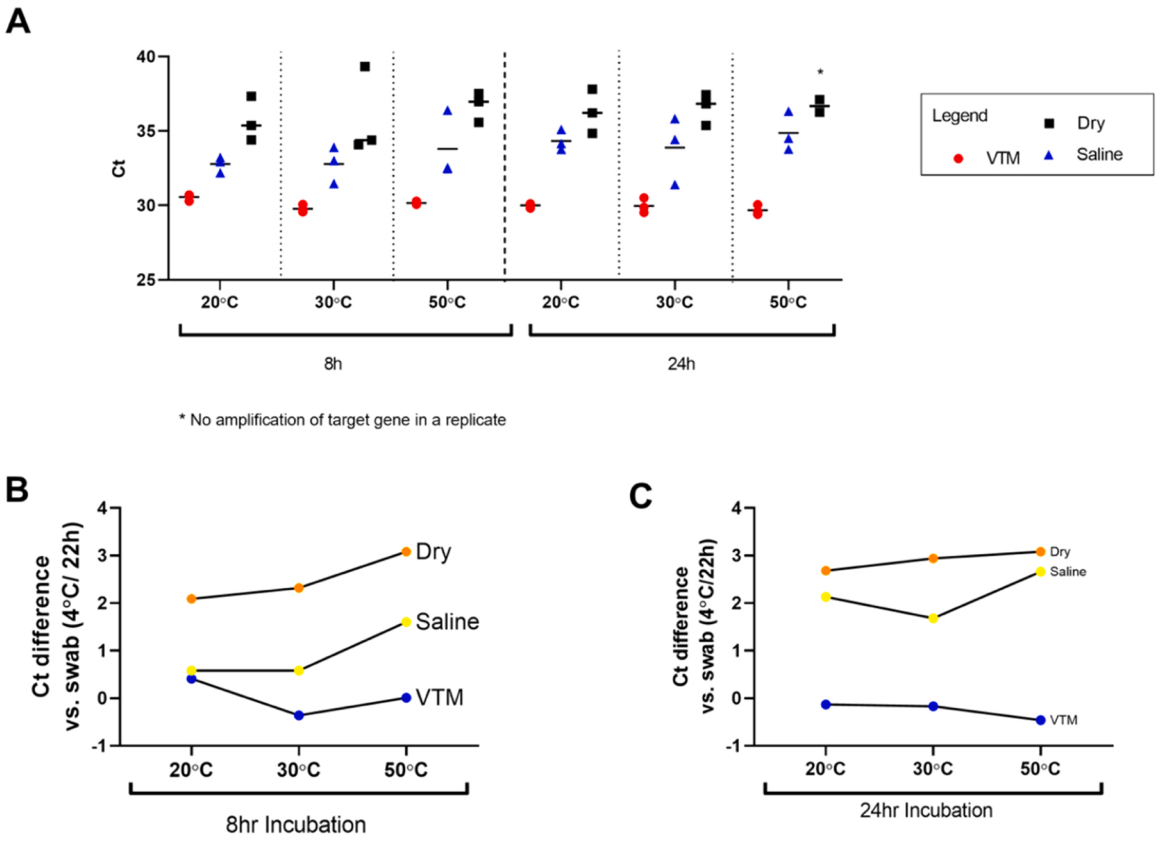

Fig. 2. A) Plots to show the Ct values of the three replicates for each swab type at increasing temperatures and different time points. Swabs in VTM are represented with a red circle, saline with a blue triangle and dry swabs with a black square. Asterisks mark where there was no SARS-CoV-2 RNA in a replicate. 2 A shows the Ct values for the testing of swabs in VTM, swabs in saline and dry swabs at 20, 30 and 50 °C at 8 h or 24 h. B) Graph to show the difference in Ct value for the different swab types stored at each temperature for 8 h compared to a sample with the same type of media kept at 4 °C for 22 h. Each point represents a different temperature, and each line represents a different swab type. C) Graph to show the difference in Ct value for the different swab types stored at each temperature for 24 h compared to a sample with the same type of media kept at 4 °C for 22 h. Each point represents a different temperature, and each line represents a different swab type.

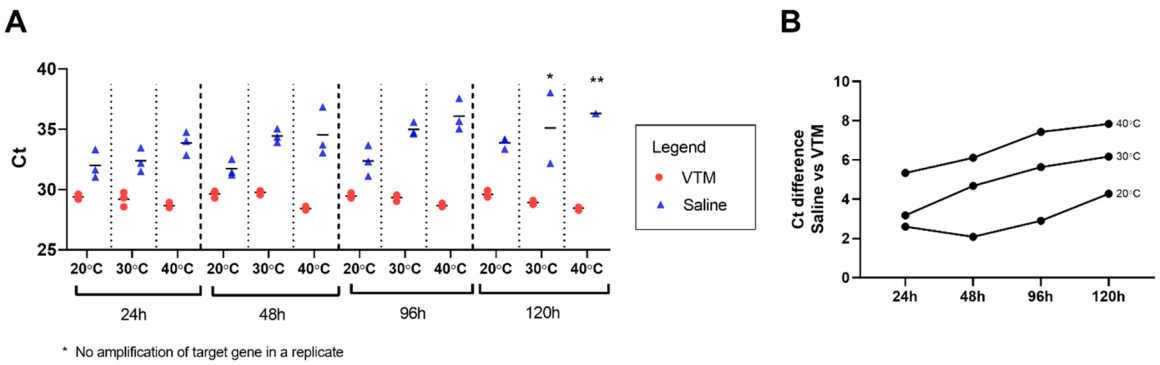

Fig. 3. A) Shows the Ct values for swabs in VTM and swabs in saline at 20, 30 and 40 °C for 24, 48, 96 and 120 h. Swabs in VTM are represented with a red circle and saline with a blue triangle. Asterisks mark where there was no SARS-CoV-2 RNA in a replicate. B) Graph displaying difference in Ct value of swabs in saline compared to the swabs subjected to the same conditions but in VTM. Each line represents storage at a different temperature and each point represents the increase in incubation time across the x axis.(For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 2
Displays the mean Ct values (of the three replicates) for each condition tested, alongside standard deviation, % CV, and Ct difference. Ct difference is compared to swab in VTM subjected to the same conditions.

Condition	Mean Ct	St Dev	%CV	Ct Difference (saline vs. VTM)
24 h				
20 °C				
VTM	29.39	0.22	0.75	
Saline	31.99	1.19	3.72	2.6
30 °C				
VTM	29.21	0.6	2.05	
Saline	32.39	1.01	3.12	3.18
40 °C				
VTM	28.66	0.23	0.80	
Saline	33.87	0.96	2.83	5.21
48 h				
20 °C				
VTM	29.63	0.29	0.98	
Saline	31.73	0.69	2.17	2.1
30 °C				
VTM	29.76	0.17	0.57	
Saline	34.44	0.56	1.63	4.68
40 °C				
VTM	28.42	0.15	0.53	
Saline	34.53	2.04	5.91	6.11
96 h				
20 °C				
VTM	29.47	0.22	0.75	
Saline	32.37	1.29	3.99	2.9
30 °C				
VTM	29.34	0.28	0.95	
Saline	34.98	0.54	1.54	5.64
40 °C				
VTM	28.66	0.17	0.59	
Saline	36.09	1.31	3.63	7.43
120 h				
20 °C				
VTM	29.6	0.28	0.08	
Saline	33.88	0.45	10.92	4.28
30 °C				
VTM	28.93	0.16	0.03	
Saline*	35.1	4.15	17.22	6.17
40 °C				
VTM	28.46	0.16	0.02	
Saline* *	36.3	N/A	N/A	7.84

* Sample not detected
**2/3 samples not detected

compared with VTM when stored at room temperature (Parikh et al., 2021). It is therefore of interest to understand how different temperatures (higher temperatures to simulate warmer climates without a cold chain) and storage times could affect the stability of dry swabs. VTM on the other hand is a universally accepted media for SARS-CoV-2 testing and is widely used but it is still suggested to transport samples using a cooling chain. This is expensive and not always feasible, and as noted may not be appropriate for unsupervised use in home-testing. Therefore, if higher temperatures and longer storage times do not affect the stability of SARS-CoV-2 RNA and consequently clinical diagnoses, then these practices can be modified to be more cost-effective and make testing more accessible.

Despite the apparent stability of SARS-CoV-2 in the environment and on surfaces with virus remaining detectable on plastic and stainless steel for up to 72 h (Morris et al., 2020) this study shows evidence that swab storage in normal saline or in no media (dry swab) is detrimental to SARS-CoV-2 RNA stability particularly over longer periods of time and at higher temperatures. Data obtained here show that SARS-CoV-2 viral RNA is more stable in VTM than in normal saline or dry swabs. In fact, RNA recovery from VTM varies very little even when held at 40 °C for 120 h (1.34 Ct). This is consistent with other studies that have found that virus on swabs stored in VTM is stable for long periods and at high temperatures. Dzung et al. (2021) found samples with a Ct between 25 and 30 cycles (moderately high viral loads) in VTM to remain stable for 21 days at 35 °C (Dzung et al., 2021).

Although our findings on storage in VTM or normal saline concord with others (Radbel et al., 2020; Summer et al., 2021) different conclusions may be drawn. Rogers and colleagues found an increase in Ct (i.e., a loss of sensitivity) with higher temperatures and longer duration of storage (Rogers et al., 2020) but concluded that the qualitative detection of SARS-CoV-2 and therefore the hypothetical clinical diagnosis would remain the same. Clearly, this is true for high viral load but any level of RNA degradation would eventually lead to a point at which virus that should be detected is not detected. Therefore here, lower titres of virus were used so that if sensitivity decreased we would detect any impact on the final qualitative result and diagnosis. Indeed, half of samples (3/6) in normal saline kept at 30 °C and 40 °C for 120hrs prior to processing had no detectable SARS-CoV-2 RNA and thus a false negative result would have been reported. The samples handled the same way but in VTM instead of saline had an average Ct value of 28. It can be argued that a storage time of 120 h (5 days) prior to processing is unrealistic in a clinical setting and such high temperatures, at least in temperate climates, are unlikely to be reached. However, there is a need for universal testing for SARS-CoV-2 and in some tropical countries, samples may have to be transported a long way as local laboratories may not be available, and at high temperatures due to the cost associated with maintenance of a cold chain. Even in temperate climates the interior of parked vehicles in direct sunlight can rise rapidly and exceed 50 °C (Wang et al., 2021). Even when samples were held at 30 °C for 24 h (a possible scenario in any region) there is a marked increase in Ct with saline vs VTM (increase of 3.18 cycles) that could lead to a false negative result.

The B.1.617.2 (Delta) SARS-CoV-2 variant was until mid- to late December 2021 the dominant strain in the UK (Gisaid, 2021) and has been shown to be associated with higher viral loads (lower Ct values) with a Ct < 30 being maintained for longer than previous strains (Ong et al., 2021) (Ong et al., 2021). Therefore, in most symptomatic cases the loss of sensitivity using swabs in saline compared to VTM would likely not be clinically significant. However, with detecting new infections (the titre may be low and rising prior to onset of symptoms) or if a test is sought towards the end of the infection to determine persistent infection this could have an impact on diagnosis (Kucirka et al., 2020). With the aim to limit exposure and spread of the virus, any loss of sensitivity with different methods leading to a false negative report could potentially have important consequences. Therefore, in clinical settings the continued use of VTM as the swab storage media is advised and reliance on the cold chain may not be necessary. In the context of large-scale surveillance of SARS-CoV-2 at the population level as in the REACT-1 study where home testing is used, this may not be practicable or desirable, however, particularly if there are concerns about potential toxicity of the VTM. A limitation of this study is the use of a cultured isolate of B.1.617.2 (Delta) SARS-CoV-2 rather than primary isolates but this allowed standardisation of virus between conditions. A further limitation is that delta strain has been replaced at time of submission with omicron strain (B.1.1.529). Future studies should include comparison between cultured and circulating primary isolates.

5. Conclusion

Importantly, from a research perspective, differences in collection methods may result in differences in the results both in the detection of cases with low viral load and when comparing viral load and thus the expected infectiousness of the virus. This is pertinent as new strains of SARS-CoV-2 emerge and when comparing results from different periods and between studies or infection before and after vaccination. Within the context of national surveillance of infections as in the REACT-1 study, such differences may affect comparisons of prevalence across different rounds of data collection but would not be expected to bias within-round trends.

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CRediT authorship contribution statement

Lucy Mosscrop: Writing – original draft, Conceptualization, Methodology, Investigation, Visualisation. **Patricia Watber:** Investigation, Writing – review & editing. **Paul Elliot:** Resources, Funding acquisition, Writing – review & editing. **Graham Cooke:** Writing – review & editing. **Wendy Barclay:** Resources, Writing – review & editing. **Paul S. Freemont:** Resources, Writing – review & editing. **Carolina Rosadas:** Investigation, Visualisation, Writing – review & editing. **Graham P. Taylor:** Supervision, Writing – review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data Availability

Data will be made available on request.

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