Survival of SARS-CoV-2 on food surfaces: Materials and Methods

5.1 Materials and Methods

The UK's Advisory Committee on Dangerous Pathogens (ACDP) has agreed on the provisional classification of SARS-CoV-2 as a hazard group 3 pathogen, which means that by default it must be handled at Containment Level 3. All viral work with the Hazard Group 3 coronavirus was performed in a Class III Microbiological Safety Cabinet in an HSE-approved Containment Level 3 laboratory at the University of Southampton.

5.1.1 Cell line, viral strain and cell culture media

The kidney cell line, VERO E6, were supplied by from UK Heath Security Agency (UKHSA). For many years, Vero cells and derivatives, have been used extensively for coronavirus cell culture research, in particular the study of SARS-CoV-like viruses. In 2020, Ogando et al., (Ogando, 2020) and many others, reported that they used Vero E6 cells to isolate, propagate and study SARS-CoV-2. This may be due to a high expression level of the ACE-2 receptor that is used by both SARS-CoV-2 and SARS-CoV.

Cells were grown at 37°C and 5% CO2 in culture medium composed of 500 ml Dulbecco's Minimal Essential Medium (DMEM) supplemented with 1% (v/v) GlutaMax-1, 1% (v/v) nonessential amino acids, 500 U/ml Penicillin, 500 µg/ml Streptomycin, and 10% (v/v) foetal bovine serum (all Thermo Fisher, UK). Cells were passaged twice a week with 0.2 – 2 ml (depending on flask size) Trypsin-EDTA (0.25%) (Thermo Fisher, UK) for 10 minutes at 37°C. Once detached the cells were centrifuged at 1800 rpm for 3 minutes, before being resuspended in fresh culture medium. Cells were grown in 25 ml, 75 ml or 175 ml flasks, depending on numbers of cells required. Cells were not used beyond passage 30 (P30) (which occurred before the onset of senescence, but susceptibility to infection diminished greatly from P30).

The human coronavirus SARS-CoV-2 strain B.1.1.7 VUI-202012/01 (the first "Variant Under Investigation" in December 2020), was obtained UKHSA. It should be noted that the results described in this report are only valid for this strain. The medium used for propagating viral stocks composed of 500 ml DMEM, 1% (v/v) GlutaMax-1, 13 ml HEPES (1M), 500 U/ml Penicillin: 500 µg/ml Streptomycin and 4% (v/v) foetal bovine serum (all Thermo Fisher, UK). SARS-CoV-2 virus stocks were prepared was by infecting Vero E6 at multiplicity of infection (MOI) of 0.01 for 4 to 7 days until a significant cytopathic effect (CPE) was observed. Infected cell supernatant was stored at -80°C.

Virus was assayed for infectious virus survival by plaque assay in 12-well plates, using Infection Medium composed of 500 ml DMEM, 1% (v/v) GlutaMax-1, 13 ml HEPES (1M); 500 U/ml Penicillin; 500 µg/ml Streptomycin and 2.5 µg/ml Amphotericin B (Thermo Fisher, UK). The plaque assay was overlaid with Avicel Overlay. Avicel Overlay consisted of a 2:1:1 mixture of 2X

Overlay Medium: 2.4% (w/v) Avicel in sterile water: sterile water (all Sigma Aldrich, UK). Overlay Medium (2X) was composed of 10X DMEM; 7.5% (v/v) sodium bicarbonate; 2% (v/v) GlutaMax-1; 1000 U/ml Penicillin, 1000 µg/ml Streptomycin; 13 ml HEPES (1M); 10% (v/v) foetal bovine serum and 147 ml sterile water (Thermo Fisher, UK) to make a final volume of 250 ml. The virus titre of different batches was very variable, at between 5 x 104 – 6 x 105 PFU/ml. SARS-CoV-2 is not a high titre virus so we could not produce a high level of virus and then dilute it down for testing, instead we used what we had produced for each batch. We do not have the facilities in the high containment CL3 laboratory to concentrate the virus further. In our initial tests we used up to 5000 PFU for each test; in later tests we used up to 10,500 PFU/test.

5.1.2 Preparation of different temperature and relative humidity conditions

We used 21°C +/- 3°C as the standard ambient temperature and 53% +/-3% relative humidity (RH). These are the temperature and humidity levels recorded inside the high containment laboratory, which has essential equipment running and does not have air conditioning. We used 6°C +/- 1°C and 53% RH+/-3% or 40% RH+/-3% as the standard chill temperature and relative humidity, as recorded inside the refrigerator in our high containment laboratory using a Fisherbrand™ Traceable™ thermometer/humidity monitor (Fisher Scientific, UK). These conditions were used as a baseline for further tests and measured every 24 hours.

RH of 20% and 80% were chosen as the extremes of RH found in supermarkets (Woradechjumroen et al., 2014). To obtain defined RH of 20%, 50% and 80% (Casanova et al., 2010; de Goffau et al., 2009), saturated solutions were made by adding more than the solubility weights provided by the manufacturer. Each saturated solution was placed in an unlidded Petri dish, inside a sealed lunch box at room temperature (ambient) and in the refrigerator (chill) temperatures before recording the actual RH using a Fisherbrand™ Traceable™ Thermometer/Clock/Humidity Monitor which was left in situ during the experiments. Fresh saturated solutions were prepared for each food and food packaging tested. The chemicals used and the actual RH obtained by using each saturated solution are shown in Table 1. To note, supply problems meant that we sometimes had to use the different chemicals to create the same relative humidity conditions (as shown in Table 1).

Table 1 Chemicals used to create different relative humidity conditions

Chemical	Relative humidity obtained
Saturated sodium chloride solubility approx. 37 g/100 ml	80% +/- 3%
Saturated ammonium chloride solubility approx. 10 g/100 ml	80% +/- 3%
Saturated magnesium nitrate solubility approx. 71g/100 ml	50% +/- 3%
Calcium sulphate solubility approx. 0.2 g/100 ml	20% +/- 3%

5.1.3 Preparation of mucin

Some experiments on food packaging were conducted in the presence of mucin, namely aluminium cans, PET1 bottles and composite drinks carton, as these products involve direct mouth contact for consumption of the product. These tests were conducted in the presence of mucin (Sigma Aldrich, UK) at the 0.1% (w/v) concentration as used by Glenister et al. (1988). One gram of mucin was dissolved in 100 ml of water at 80°C for one hour and then autoclaved at 121°C for 15 min; it was subsequently diluted to 0.1% (w/v) in the SARS-CoV-2 virus stock.

5.1.4 Determination of optimum protocol for virus recovery

To determine the optimal protocol for virus recovery from each food, we tested and compared 3 methods, namely pulsification, vortexing with beads and swabbing. We tested each method for recovery of virus from broccoli, peppers, apples, raspberries, white bread crust, brown bread crust, croissant, pain au chocolat, ham, cheese and olives. Results are shown in 6.1 and 6.2.

We used the Pulsifier (Pulsifier II, Microgen Bioproduct%s Ltd, Camberley, UK) as described by Highmore et al., 2018. The Pulsifier method is a gentle, but very effective method for recovery of bacteria and viruses from foods (ISO 7218; Kang et al., 2007; Sanchez et al., 2012). The food samples were placed in a plastic bag in 10 ml Infection Medium, placed in the Pulsifier which was run for 30 seconds. The Pulsifier utilises a high frequency oscillating metal ring (approximately 3000 reciprocations/minute) to beat the plastic bag thus forcing the virus off the food surface.

Despite being a vigorous method, our experience recovering virus from metal coupons with beads/vortexing, shows that both viral recovery and virus viability are high (Warnes et al., 2015) and therefore this method is suitable for virus recovery from foods (Goyal and Aboubakr, 2016). Food samples were vortexed with a Grant PV-1 vortex mixer at 3000 rpm for 60 seconds in 10 ml Infection Medium containing 2 g of glass beads with diameter of 2 mm glass beads (Fisher Scientific, UK).

We also tested swabbing on all foods in this study. Although in some previous studies, swabbing was shown to lead to very poor recovery of viruses from complex foods (Moore and Griffith, 2007), a recent study showed that for recovery of SARS-CoV-2 from food surfaces, nylon flocked swabs were a valid tool for detection, allowing improved sample absorption and high quantity release of viruses from the sample surface (Arnaboldi et al., 2022). Swabbing was therefore performed using nylon tipped swabs (TCS Swabs, UK). The swab was dipped into Infection Medium before swabbing the sample. The swab was rotated during the swabbing and the surface swabbed in two directions to ensure the whole surface was swabbed. For foods with complex shapes, the whole or piece of individual food was carefully swabbed all over the surface. The swab was placed into 10 ml Infection Medium containing glass beads and vortexed for 30 seconds.

5.1.5 Preparation of foods and food packaging for testing

The foods tested were selected as they are commonly found loose on supermarkets shelves, they may be difficult to wash, and they are often consumed without any further processing i.e. cooking. The food packaging materials were selected as they are the most commonly used food packaging materials or consumption of the product may involve direct mouth contact. The foods studied for ascertaining baseline parameters were fresh vegetables (broccoli and red pepper), fresh fruit (apples and raspberries), baked products (croissants, pain au chocolat, brown bread crust and white bread crust), and delicatessen items (cheddar cheese, sliced ham, olives and olive brine). All tests were performed in triplicate. Each food type was left intact (raspberry and green olives); or cut into approx. 5 g pieces (broccoli, pepper, ham, cheese, brown bread crust, white bread crust, croissant, pain au chocolat); or cut into 3 cm x 3 cm pieces (apple) to make handling of the foods more practicable in the confines of the CL3 cabinet, as well as to perform vortexing with beads and pulsification protocols. The size and weight of each food was chosen to allow ease of handling within the confines of the Class III Microbiological Safety Cabinet. Three pieces or individual whole foods were used at each time point. 5 ml volumes of the brine that surrounded the olives were also tested. Brine is a solution of salt and water, with an extremely high salinity. It has been used historically in food production as a preservative since salt inhibits the growth of microorganisms. The brine tested was at a concentration of 4% NaCl and a pH of 4.5.

The food packaging studied were polyethylene terephthalate (PET1) containers (PET1 bottles and PET1 trays); aluminium cans and composite drinks cartons. Food packaging was cut into approx. 1 cm x 1 cm pieces and all tests performed in triplicate using 3 randomly selected pieces

per time point. The size of each food packaging type was chosen to allow ease of handling within the confines of the Class III Microbiological Safety Cabinet.

5.1.6 Time course infectivity assay for foods artificially contaminated with SARS-CoV-2

We measured the survival of infectious virus present on food surfaces over time. All tests were performed in triplicate. Each food type was left intact (raspberry and green olives); or cut into approximately 5g pieces (broccoli, pepper, ham, cheese, brown bread crust, white bread crust, croissant, pain au chocolat); or cut into 3 cm x 3 cm pieces (apple). Three pieces of food or 3 individual foods, were used at each time point. 5 ml volumes of the brine that surrounded the olives was also tested.

Aliquots of 20 µl SARS-CoV-2 were added to each surface, to simulate respiratory droplet contact (Warnes and Keevil, 2015). A 10 µl calibrated micropipette was used to artificially contaminate the SARS-CoV-2 cell culture supernatant onto each sample surface or into the brine liquid. Aliquots of 20 µl (3 x 6.67 µl) (containing between 1000 and 5000 PFU) SARS-CoV-2 were added to the outer surfaces of each food, and immediately spread as evenly as possible with the tip of the micropipette over the entire outer surface of the sample using a back-and-forth motion. For brine, 20 µl SARS-CoV-2 was added to 5 ml brine and mixed thoroughly.

All samples, except brine, were then allowed to dry for 60 minutes at room temperature inside the high containment cabinet. Brine samples containing virus were allowed to stand for 60 minutes at room temperature inside the high containment cabinet. All samples were then placed in sealed lunch boxes at the required temperature and relative humidity.

Virus was removed from the test material at the various timepoints, using one of three methods described in 5.1.4: vortexing with beads, pulsification or swabbing. The results can be seen in Table 2.

5.1.7 Time course infectivity assay for packaging materials artificially contaminated with SARS-CoV-2

We measured the survival of infectious virus present on food packaging over time. All tests were performed in triplicate, using 3 randomly selected pieces per time point. Each food packaging piece was cut into several approx. 1 cm x 1 cm pieces; 3 pieces were used at each time point.

A 10 µl calibrated micropipette was used to artificially contaminate the SARS-CoV-2 cell culture supernatant onto each sample surface. Aliquots of 20 µl (3 x 6.67 µl) (equivalent to between 10000 and 10250 PFU, depending on viral stock used) SARS-CoV-2 were added to the outer surfaces of food packaging pieces and immediately spread as evenly as possible with the tip of the micropipette over the entire outer surface of the sample using a back-and-forth motion. Samples were allowed to dry for 60 minutes at room temperature inside the high containment cabinet and were then placed in sealed lunch boxes at the required temperature and relative humidity.

Virus was removed from the test material at the various timepoints, using the vortexing with beads method. Each time point was performed in triplicate, with an individual piece of each food packaging used for each of the triplicate samples.

5.1.8 Plaque assay

The resulting food or food packaging sample extracts were taken up in a 10 ml disposable syringe (SLS, UK) and push-filtered through a 0.45 µm low protein and extractables binding PES filter (Millipore, UK) to clarify the suspension and remove bacterial and fungal contaminants. Virus

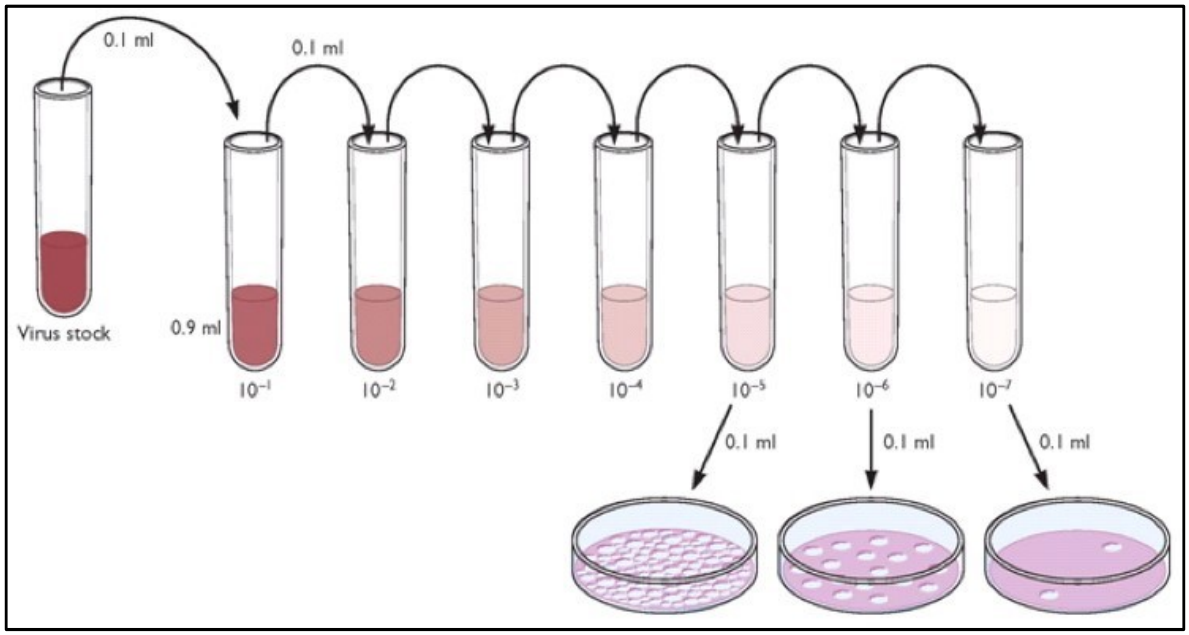
stocks and recovered virus titres were assayed for infectious virus by plaque assay (Dulbecco and Vogt, 1953), with the results described as plaque-forming units (PFU); see Figure 1.

Serial dilutions were performed if required, to ensure that countable plaques were obtained (neat, 10-1, 10-2, 10-3). Dilutions were prepared in Infection Medium, and 400 µl aliquots of each dilution plated onto confluent monolayers of Vero E6 cells that had been prepared by plating 2.5 x 105 cells in 1 ml growth medium, 24 h earlier in 12-well plates. The inoculum was removed after 60 min and replaced with 1.5 ml Avicel Overlay. The plates were incubated at 37°C and 5% CO2 in air for 3 days. The monolayers were fixed for 30 minutes in 8% (w/v) paraformaldehyde, stained with 0.1% crystal violet, allowed to dry and plaques in the monolayer enumerated as in the equation:

PFU/sample = C x D x V

Where C is the smallest number of countable plaques per well; D is sample dilution in well and V is volume (ml) of packaging sample extract. The mean limit of detection (LOD) for the assay is determined as the lowest spiking concentration that produced plaques. For the assay described here the LOD is 25 PFU/sample (Warnes et al., 2015): in this study the limit of detection for the smallest number of countable plaques per well is 1, thus C = 1; 400 µl of sample was added to each well, thus D = (1000 µl/400 µl) = 2.5; and the volume of sample extract was 10 ml, thus V = 10.
This equates to 25 PFU/sample being the mean limit of detection (LOD). At each time point, 3 independent sample tests were performed, A, B and C. For each of these independent tests the assay was performed in duplicate, resulting in 2 wells per dilution of each sample, A, B and C. Means were then calculated to give PFU/sample shown in the results Tables 4 – 155 and Figures 2 - 27. Sometimes, there were no plaques visible, meaning that there were fewer than 25 infectious particles produced in the assay, (i.e. fewer than 25 PFU remained on the sample), giving a mean of <25 PFU/sample. In these cases, 25 PFU/sample have been plotted on the graphs and <25 PFU/sample recorded in the table. A blue line (?) on the graphs shows the LOD; all PFU below the LOD are shown as 25 PFU, since values below 25 PFU could not be calculated.

Figure 1 Dilution series for determining PFU. Adapted from Viral Titering-Plaque Assay Protocol, 2020



5.1.9 Statistical analysis

Where appropriate, the data sets for the food and food packaging tests are expressed as mean +/- standard error of the mean (SEM) and error bars shown on the graphs represent the SEM. The data sets were evaluated for statistical differences using one-way analysis of variance (ANOVA) or two-way analysis of variance (ANOVA) followed by Tukey's multiple comparisons post hoc test, using GraphPad Prism (version 9.3.1, GraphPad Software, USA). Statistical significance was indicated where the p-value was less than 0.05 ($p<0.05$). ANOVA is a statistical test that is used to determine if there is a statistical difference between the means of two or more groups of data. If the ANOVA results indicate statistical significance, post-hoc analysis is performed. Post-hoc (in Latin 'after this') testing is used when a statistical test of three or more samples is statistically significant, and you wish to determine which individual samples are different from one another. The main ANOVA test tells you only that one or more of the samples is different from one or more of the other samples. In Tukey's post-hoc test, every mean is compared with the mean of all other groups.