Australia's National
Science Agency

Dairy Australia Report – Lumpy Skin Disease transmission studies in milk

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1 Executive summary

Since detection of Lumpy Skin Disease Virus (LSDV)in Indonesia in 2022, the risk of an incursion into Australia has significantly increased. Various preparedness or awareness activities have been made possible by the lifting of restrictions which previously prevented the import of live virus strains into the Australian Centre for Disease Prevention. CSIRO was approached by Dairy Australia to determine the effect of milk pasteurisation (72°C for 15 seconds) on the viability of LSDV in contaminated milk. To minimise heating variables between an industrial/commercial and laboratory setting, raw milk was pre-heated to either 72°C, 75°C or 80°C, then spiked with various concentrations of LSDV and held for either 7.5, 15 or 30 seconds, then immediately chilled. The pasteurised milk was then titrated to determine levels of viable virus remaining in the sample.

Results from the pasteurisation studies have demonstrated that no viable virus was detectable in any combination of time, temperature or virus concentration in milk.

2 Introduction

Lumpy skin disease virus (LSDV) is a contagious viral disease of cattle, buffalo, and other bovines. It is caused by a capripoxvirus, which is closely related to the viruses that cause sheep pox and goat pox. LSDV is characterised by the formation of nodules, or lumps, on the skin of infected animals. The disease is not fatal, but it can cause significant economic losses to farmers due to reduced milk production, weight loss, and increased mortality rates of calves.

Since its initial detection in Indonesia in early 2022, LSDV has been confirmed in 15 provinces such as Aceh, North Sumatra, West Sumatra, South Sumatra, Riau, Jambi, Bengkulu, Lampung, Banten, West Java, Central Java, East Java, Yogyakarta, and Central Kalimantan Province.

Given the role of insect vectors in the spread of LSDV, the risk of an outbreak in Australia has now increased significantly. Dairy Australia approached CSIRO to provide data on inactivation of LSDV in milk to inform any subsequent risk assessments concerning dairy products. More specifically, Dairy Australia were interested in inactivation of LSDV in pasteurisation of potentially contaminated milk. Australia uses high temperature, short time (HTST) pasteurisation (72°C for 15 seconds) to treat milk destined for human consumption.

The aim of this project was to determine the effects of pasteurisation on LSDV contaminated milk. The project was designed to cover a broad range of temperatures (72°C, 75°C and 80°C), times (7.5, 15 and 30 seconds) and virus concentrations (10^4, 10^5 and 10^6 $TCID_{50}$) in raw milk.

Note: A glossary of terms is located on page 29 for acronyms referred to within this document.

3 Methods

3.1 Virus

The Lumpy skin disease virus strain utilised in this study was Israel 2007 provided by the Pirbright Institute (UK). The virus was passaged in Madin-Darby Bovine Kidney (MDBK) cells (ATCC CCL 22) using Dulbecco's Modified Eagle Medium (DMEM) supplemented with Penicillin, Streptomycin, amphotericin B, non-essential amino acids and 10% fetal calf serum (FCS). For this project virus was purified and pelleted via ultracentrifugation at 100,000 x *g* for 90 minutes over a 36% sucrose cushion. The virus was resuspended in STE buffer (Sodium Chloride, TRIS-EDTA buffer pH 7.4) and stored at -80°C. The virus stock was titrated via $TCID_{50}$ and was determined to be $1.34x10^8$/mL via Spearman-Kärber method.

3.2 Milk

Raw milk was sourced from the southwestern region of Victoria. Milk was sourced from a single farm direct from holding vats prior to any homogenisation or pasteurisation. To ensure consistency between experiments, a single batch of milk was frozen into small aliquots and 1 aliquot was thawed on the day of each experiment. The batch of milk used in the study contained 4.61% fat, 3.850% protein, a milk solids composition of 8.46% and a bulk milk cell count of 190.

3.3 Pasteurisation

To simulate the pasteurisation process, sealed plastic tubes with 250µL raw milk were placed into a heat block set to either 72°C, 75°C or 80°C. To monitor temperatures during the simulated pasteurisation process, a Graphtec GL840 data logger fitted with thermocouples was used. Thermocouples were placed into the heat block (to confirm the temperature of the equipment) and a control tube filled with raw milk (to monitor temperature within tubes). Tubes to be spiked with virus during the experiment were treated in an identical manner to the control tube. Two separate methods were initially trialled for the pasteurisation experiments. The first method involved virus being added to the chilled raw milk, then placed into the heat block, once the control tube reached the desired temperature the timing was started. Once the set pasteurisation time was achieved the tubes were placed in an ice bath to instantly chill. The second method involved tubes containing only milk being added to the heat block, and once the control tube reached the desired temperature, virus was added to the tubes, and timing commencing immediately. Once the set times were reached, the tubes were chilled on ice to stop the pasteurisation process. To ensure robustness of data, all variables (time, temperature and virus concentration) were performed in triplicate.

3.4 $TCID_{50}$ and Isolation

To determine the titre of any infectious virus remaining in the milk samples, a $TCID_{50}$ assay was performed for each sample. The assay was performed with 4 replicates. The $TCID_{50}$ assay was carried out by performing a series of 10-fold dilutions of the virus/milk mix to quantify the amount of infectious virus remaining in the sample. Serial dilutions of the sample were added to a susceptible cell line (MDBK) and any cytopathic effect visualised microscopically. Positive control samples (virus in milk, held at 4°C) were assayed for each concentration of virus for each experiment.

Raw milk contains significant proportions of fats and proteins and was toxic to cell monolayers in a $TCID_{50}$ and was not able to be reliably tested. To reduce the toxicity of the milk, samples were passed through 40kDa Zeba desalting columns prior to setting up $TCID_{50}$, positive and negative control samples were also passed through columns.

In addition to $TCID_{50}$, isolation on susceptible monolayers was also attempted to provide the best opportunity to isolate any viable virus that may still be present in the sample. Equal volumes of sample were used for $TCID_{50}$ and Isolation. Isolations were performed as follows; pasteurised, column purified sample was inoculated onto 80% confluent monolayer of MDBK cells in a $75cm^2$ flask. Flasks were incubated for 7 days and observed for signs of CPE on multiple occasions. At the end of the 7 days, flasks were frozen/thawed 3 times, then 1mL of the supernatant was inoculated onto a fresh flask of MDBK cells and observed for a further 7 days. The isolation was considered negative if no CPE was observed at the completion of the 2^{nd} passage.

With the combination of volume used in the TCID50, and the flasks for isolation, the limit of detection was determined to be 0.8 log (TCID50/mL).

4 Preliminary studies

4.1 Bacterial load – Milk

To assess the potential role of excess bacteria in milk samples, raw milk was titrated in a mock $TCID_{50}$ assay, cellular toxicity and fungal/bacterial growth was observed at a neat and 1:10 dilution, with no morphological changes in the cell monolayer observed at the 1:100 dilution.

Passing material through Zeba spin columns and increasing the antibiotic concentration in the media reduced toxicity to the neat dilution and reduced bacterial load significantly (only sporadic wells with bacteria are observed).

4.2 Temperature parameters

While there is considerable published information regarding pasteurisation times, there is little if any information on the length of time taken for milk to reach pasteurisation temperature from 4°C. At the commencement of the project, it was unknown how long the ramping times were in a commercial/industrial setting.

Initial studies involving sealed Eppendorf tubes placed into a heat block within a biological safety cabinet. Thermocouples were placed into the centre of the tubes with 1ml of raw milk and took approximately 9m30sec to reach 72°C (fig 1), 10m10sec to reach 75°C and 10m57sec to reach 80°C however, there was over 1 minute variation between replicates for each temperature point.

When the volume was lowered to 250µL, the ramping times were significantly reduced, with the average being between 2 and 3 minutes across the 3 temperatures ranges.

Changing tube material type to stainless steel also failed to increase ramping times significantly.

CSIRO were concerned that prolonged heating times were contributing to inactivation of virus prior to the target temperature being reached. Enveloped viruses such as LSDV are particularly sensitive to high temperatures and temperatures above 56°C will contribute to viral inactivation. Preliminary data demonstrated that 1mL of ice cold milk took over 9 minutes to reach 72°, and that over 7 minutes of this time the temperature was in excess of 56°C (Fig 1.).

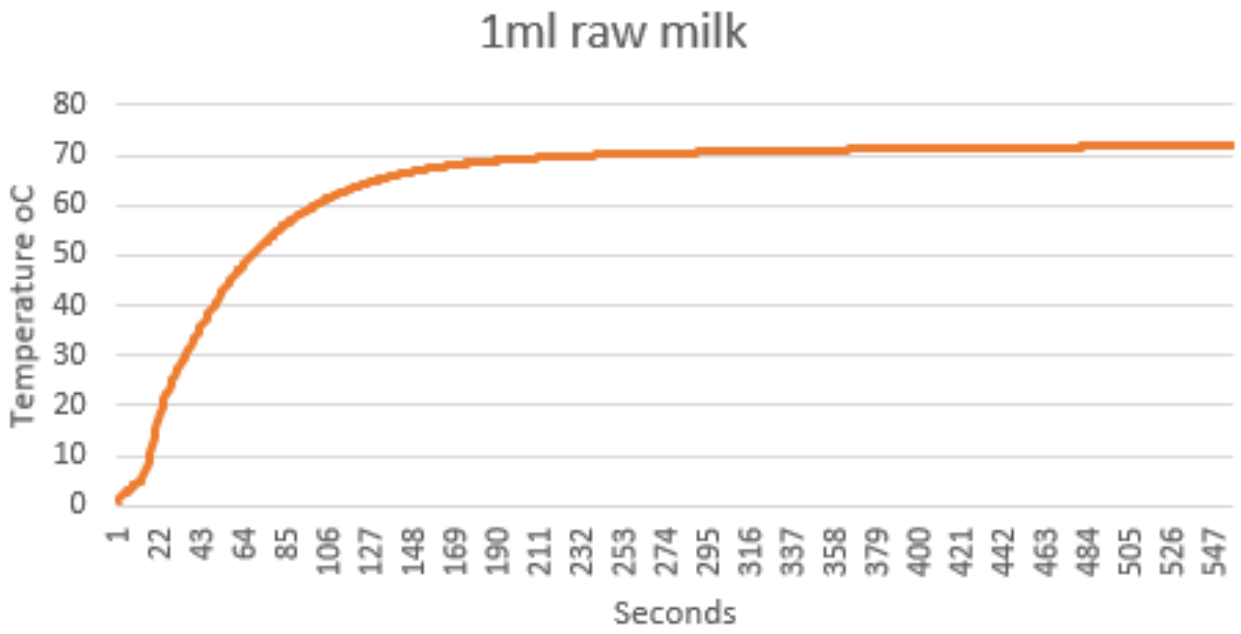


Figure 1. An example of a temperature trace showing 1mL of ice cold raw milk took over 9 minutes (540 seconds) to reach 72°C in a heat block.

Advice from Dairy Australia suggested that the ramping times from 4°C to 72°C in an industry setting was approximately 10 seconds. Following this information, it was clear an alternate approach was required. It was decided that while CSIRO could not achieve the short ramping times, the best approach would be to preheat raw milk to the required temperatures, then spike the samples with virus the moment the tubes reached temperature. The tubes would then be placed on ice following the 7.5, 15 or 30 second incubation time. This method allowed for an exact pasteurisation time and reducing any unintentional inactivation caused by a slower ramping time as seen in the previous experiments.

4.3 Pasteurisation testing method

Milk was thawed and aliquoted into 250µL tubes. To determine temperature of the milk in the tubes, a dummy tube (milk, no virus) was placed alongside the test tubes with a thermocouple connected to a Graphtec data logger. Tubes were placed into a heat block set to either 72°C, 75°C or 80°C, once the milk reached the desired temperature, virus was added to the test tubes and timing commenced immediately. At either 7.5, 15 or 30 seconds tubes were removed from the heat block and placed on ice to chill as quick as possible.

Each time/temperature combination was pooled from 2x250µL samples from individual tubes and tested in triplicate (6 x 250µL tubes per time/temperature/virus concentration variation). To reduce the toxicity and bacterial load, the milk was passed though Zeba spin columns. For $TCID_{50}$ of the sample, 222µL of each sample was titrated with 4 replicates, with the remaining sample added to a $75cm^2$ flask for isolation (to confirm presence or absence of viable virus). Two, seven-day passages were performed on the lowest stringency pasteurisation conditions (72°C for 7.5 and 15 seconds) to confirm if viable virus was detected. For all other temperature and time combinations, only 1 passage was performed. The addition of the isolation method ensures that any toxicity observed in the $TCID_{50}$ is not masking the potential of any viable virus remaining in the sample that is unable to be resolved in the initial test ($TCID_{50}$).

As LSDV is a Risk Group 3 organism, all work was conducted in a Class II Biological Safety Cabinet (BSCII).

5 Results

5.1 72°C for 7.5 seconds

Results from both $TCID_{50}$ and isolation demonstrated no viable virus remained in the milk samples that were pasteurised at 72°C for 7.5 seconds. No CPE was observed beyond toxicity in the $TCID_{50}$, and no virus like CPE was observed in the two passages of isolation on susceptible cells.

To confirm this result, Real Time RT-PCR detecting LSDV nucleic acid was performed on both first and second passages for the samples. Results from this testing showed an increasing cycle threshold (CT) value for each passage. An increase in CT value relates to a decrease in target nucleic acid, indicating that the virus is not amplifying and is non-infectious.

Results of the $TCID_{50}$ have demonstrated that no infectious virus was detectable following pasteurisation for 7.5 seconds at 72°C. Bars on the test samples of figure 3 are set at 0.8, as this is the limit of detection for the assay.

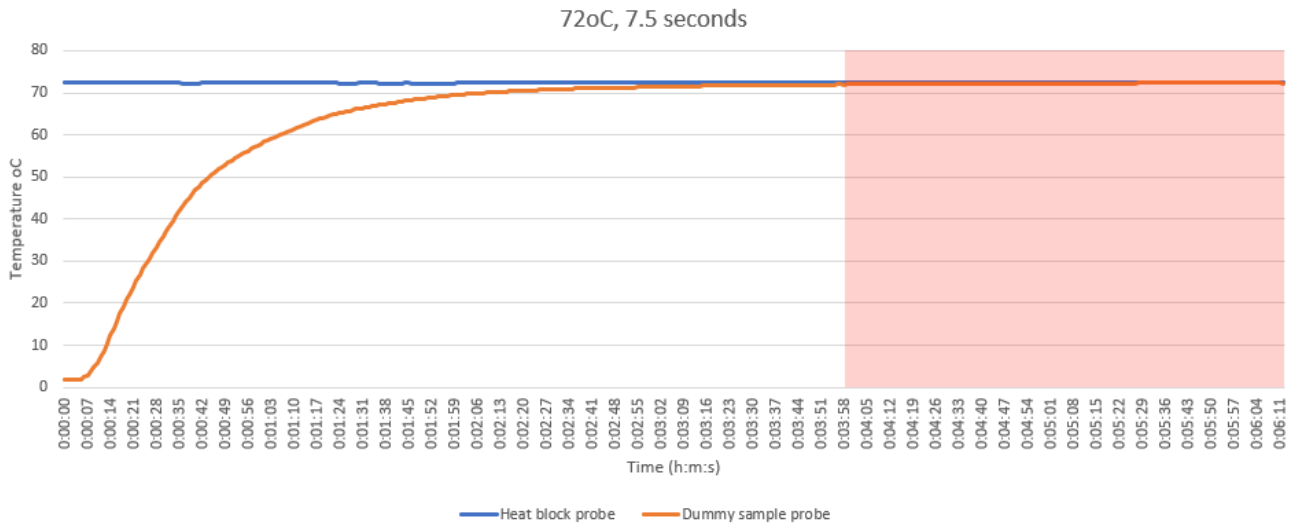


Figure 2. Temperature read out from the data logger files for the pasteurisation experiment. The blue line represents the temperature of the heat block, and the orange line represents the temperature of the dummy milk sample. The red box represents the period of time where the dummy sample reached the appropriate temperature, and spiking experiments were conducted in this time period.

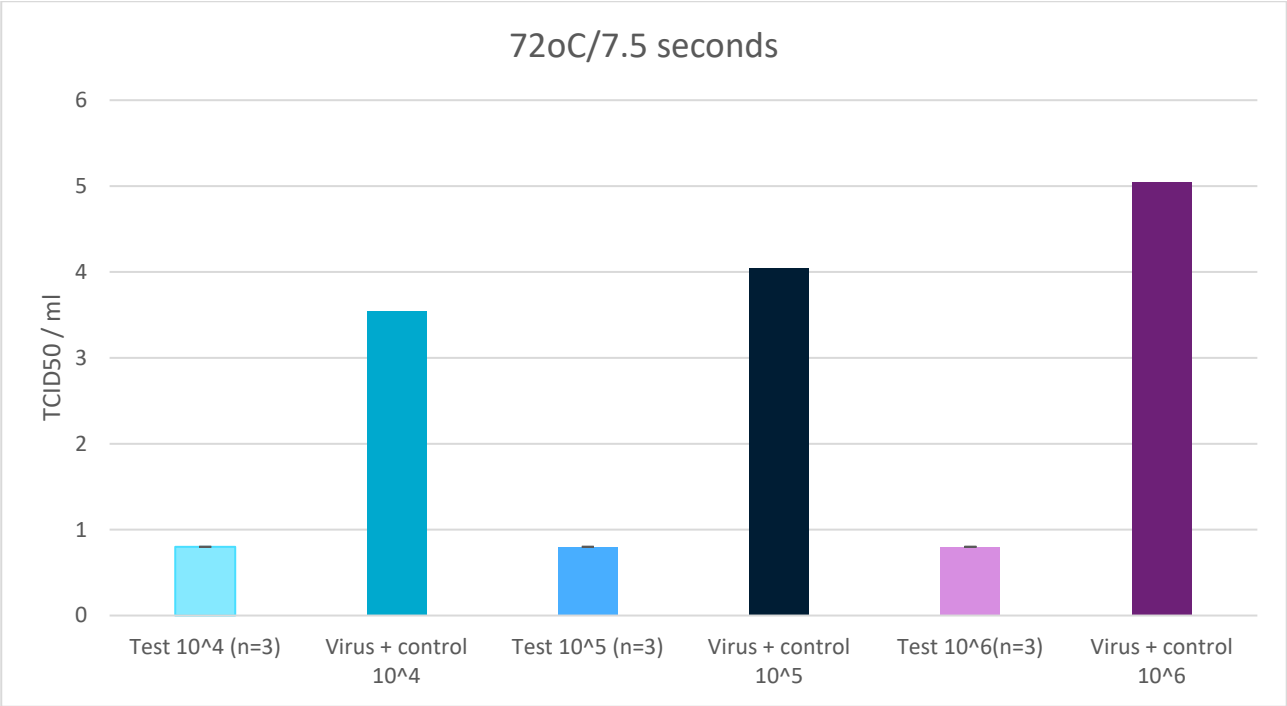


Figure 3. $TCID_{50}$ results for 72°C for 7.5 seconds – no infectious virus was detected in the 10^4, 10^5 and 10^6 samples (referred to as Test 10^4, Test 10^5 and Test 10^6, respectively). Positive controls (termed Virus + control) for each virus concentration (in milk, kept at 4°C) were performed alongside each test sample. The limit of detection for the assay is 0.8 $TCID_{50}$/mL.

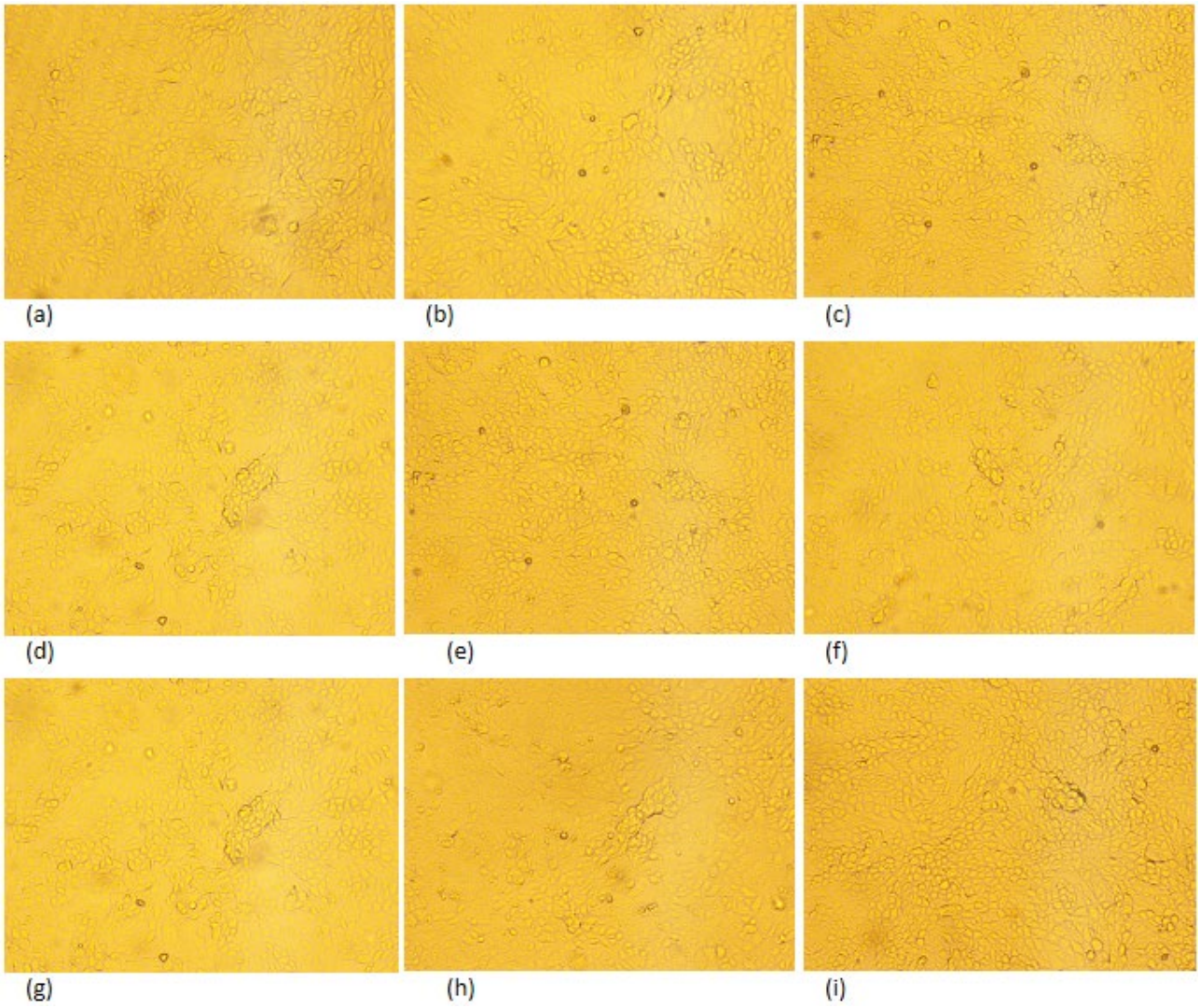


Figure 4. Cell monolayers from flasks inoculated with milk, which was spiked with virus and then pasteurised for 7.5 seconds at 72°C. Images (a), (b) and (c) represent samples spiked with 10^4 LSDV. Images (d), (e) and (f) represent samples spiked with 10^5 LSDV. Images (g), (h) and (i) represent samples spiked with 10^6 LSDV. Cell monolayers are intact and showing no signs of cytopathic effect (CPE) suggesting no viable LSDV is present in the samples.

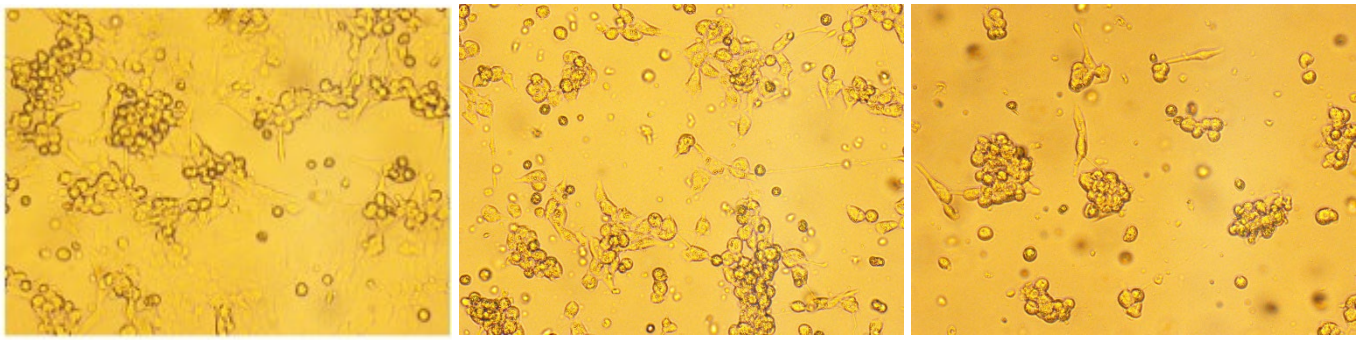


Figure 5. Cell monolayers infected with LSDV. Image (a) is inoculated with 10^4 LSDV, image (b) is inoculated with 10^5 LSDV and image (c) is inoculated with 10^6 LSDV. Typical LSDV-like CPE is observed across all three samples.

5.2 72°C for 15 seconds

Results from both $TCID_{50}$ and isolation demonstrated no viable virus remained in the milk samples that were pasteurised at 72°C for 15 seconds. No CPE was observed beyond toxicity in the $TCID_{50}$, and no virus like CPE was observed in the two passages of isolation on susceptible cells.

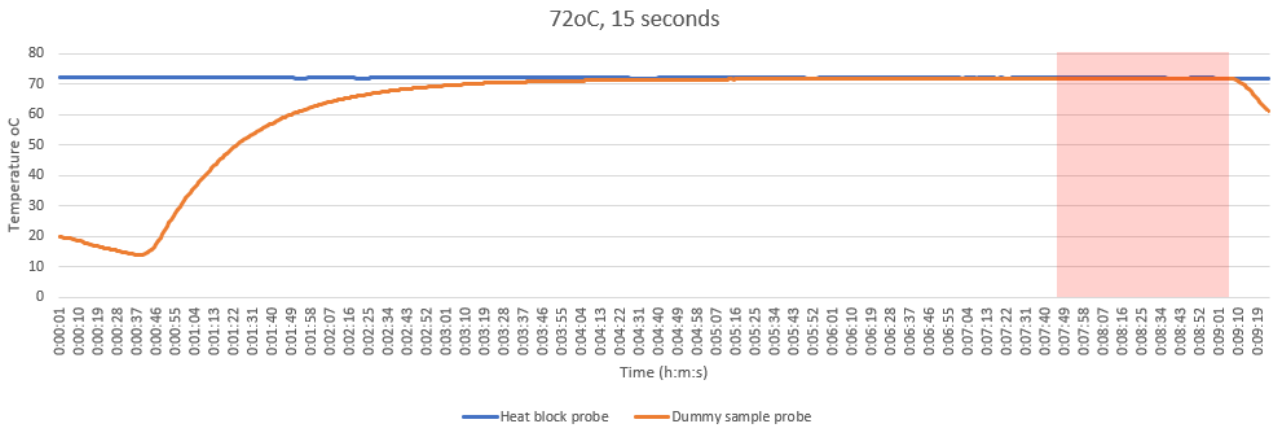


Figure 6. Temperature read out from the data logger files for the pasteurisation experiment. The blue line represents the temperature of the heat block, and the orange line represents the temperature of the dummy milk sample. The red box represents the period of time where the dummy sample reached the appropriate temperature, and spiking experiments were conducted in this time period.

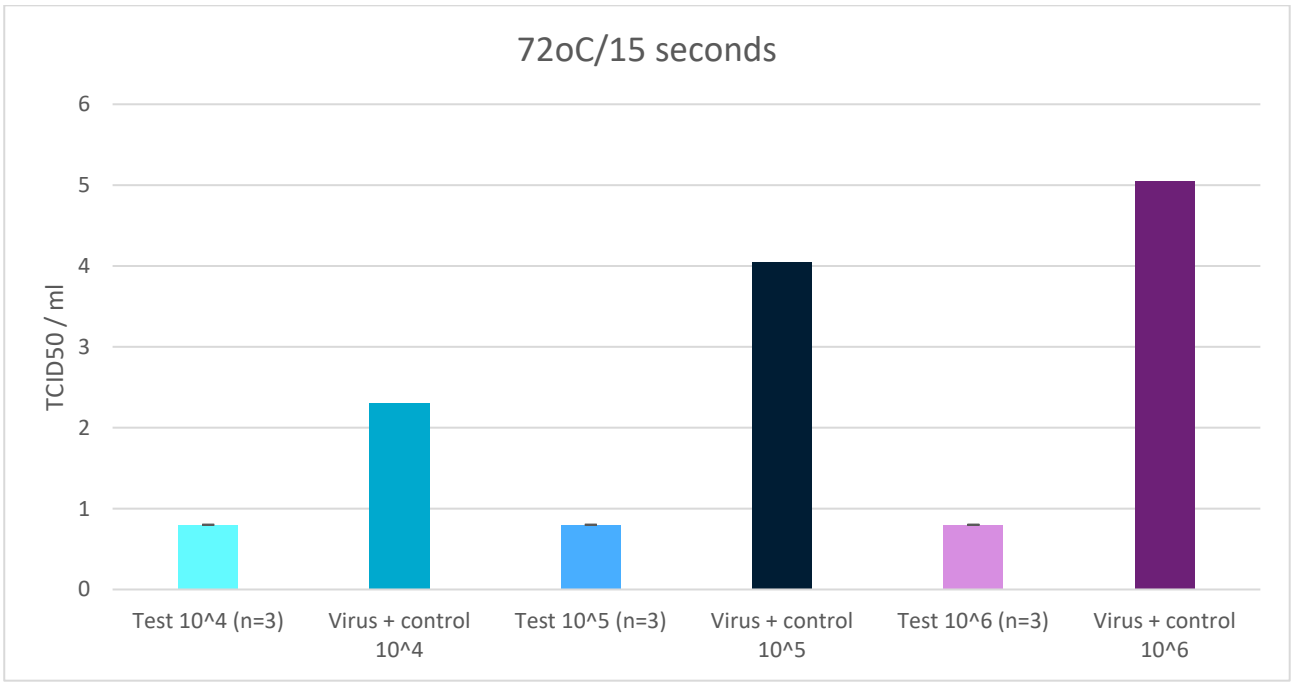


Figure 7. $TCID_{50}$ results for 72°C for 15 seconds – no infectious virus was detected in the 10^4, 10^5 and 10^6 samples (referred to as Test 10^4, Test 10^5 and Test 10^6, respectively). Positive controls (termed Virus + control) for each virus concentration (in milk, kept at 4°C) were performed alongside each test sample. The limit of detection for the assay is 0.8 $TCID_{50}$/mL.

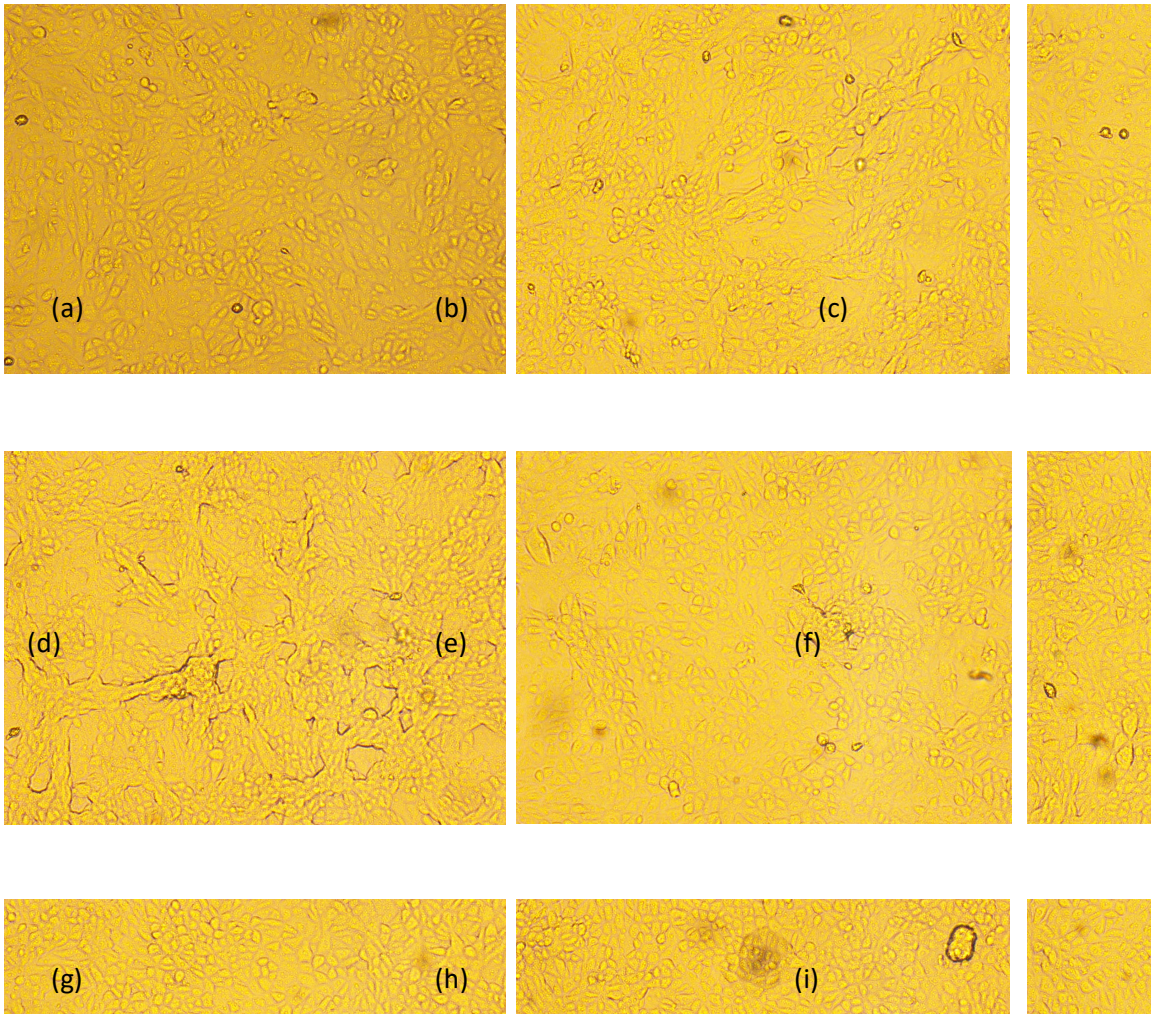
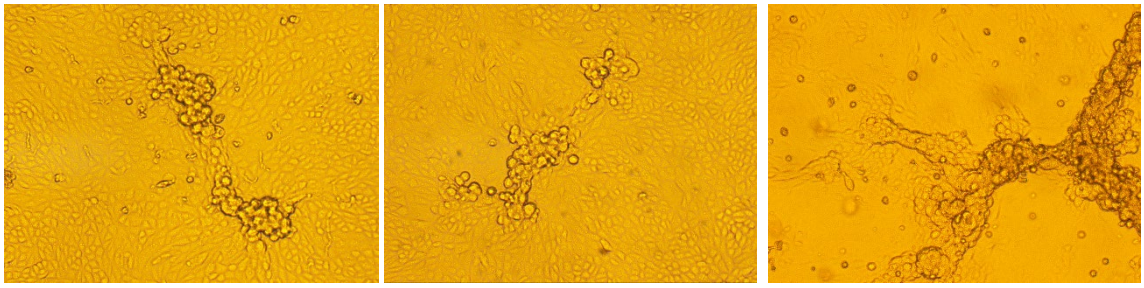


Figure 8. Cell monolayers from flasks inoculated with milk, which was spiked with virus and then pasteurised for 15 seconds at 72°C. Images (a), (b) and (c) represent samples spiked with 10^4 LSDV. Images (d), (e) and (f) represent samples spiked with 10^5 LSDV. Images (g), (h) and (i) represent samples spiked with 10^6 LSDV. Cell monolayers are intact and showing no signs of cytopathic effect (CPE) suggesting no viable LSDV is present in the samples.



(a) (b) (c)

Figure 9. Cell monolayers infected with LSDV. Image (a) is inoculated with 10^4 LSDV, image (b) is inoculated with 10^5 LSDV and image (c) is inoculated with 10^6 LSDV. Typical LSDV-like CPE is observed across all three samples.

5.3 72°C for 30 seconds

Results from both $TCID_{50}$ and isolation demonstrated no viable virus remained in the milk samples that were pasteurised at 72°C for 30 seconds. No CPE was observed beyond toxicity in the $TCID_{50}$, and no virus like CPE was observed in the two passages of isolation on susceptible cells.

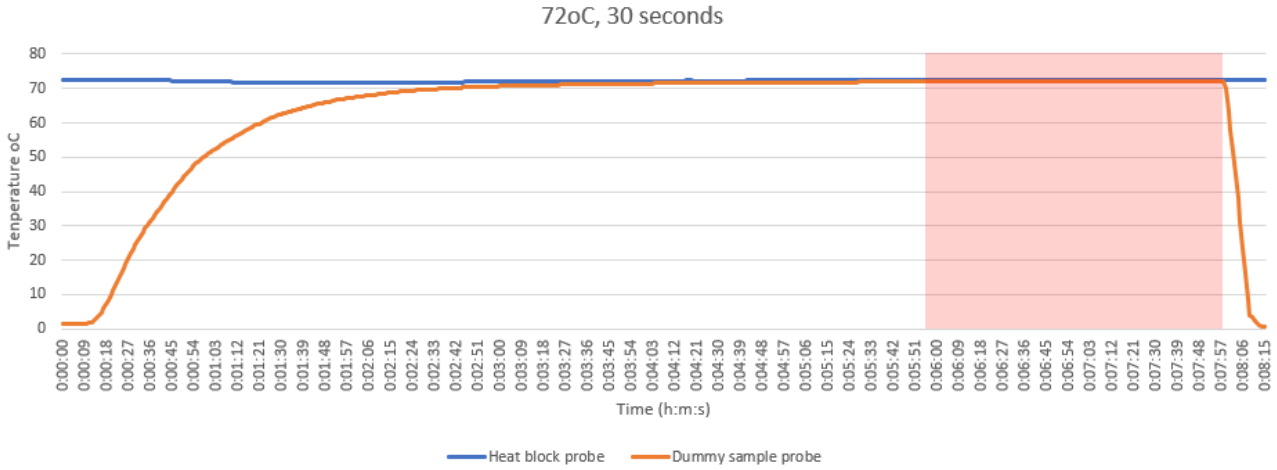


Figure 10. Temperature read out from the data logger files for the pasteurisation experiment. The blue line represents the temperature of the heat block, and the orange line represents the temperature of the dummy milk sample. The red box represents the period of time where the dummy sample reached the appropriate temperature, and spiking experiments were conducted in this time period.

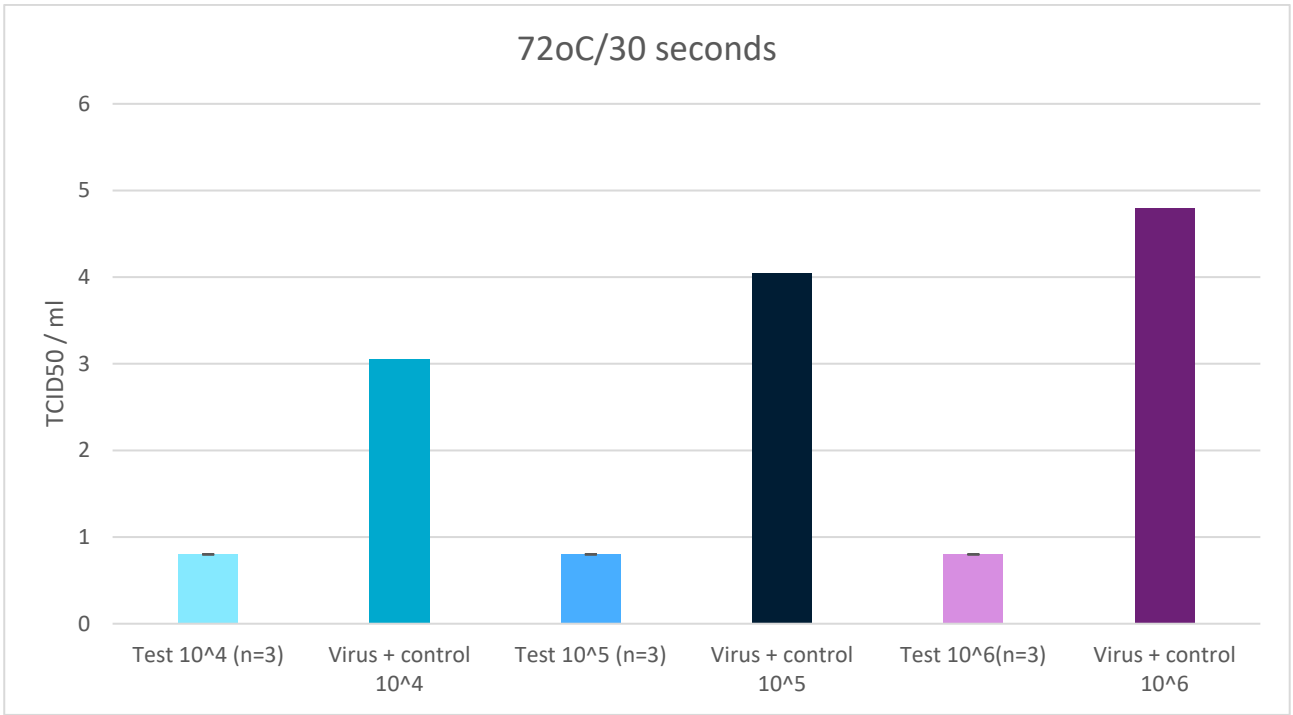


Figure 11. $TCID_{50}$ results for 72°C for 30 seconds – no infectious virus was detected in the 10^4, 10^5 and 10^6 samples (referred to as Test 10^4, Test 10^5 and Test 10^6, respectively). Positive controls (termed Virus + control) for each virus concentration (in milk, kept at 4°C) were performed alongside each test sample. The limit of detection for the assay is 0.8 $TCID_{50}$/mL.

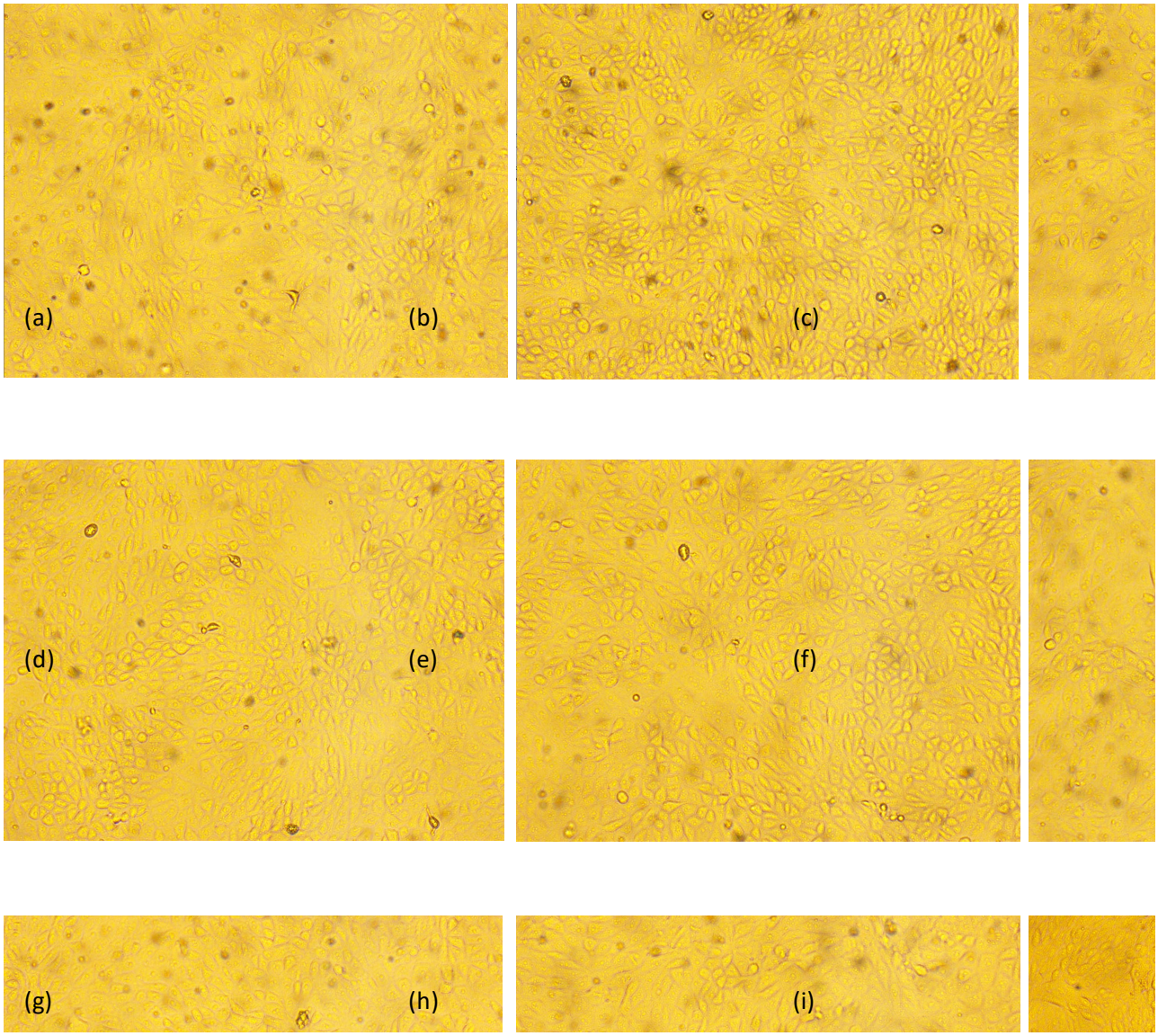


Figure 12. Cell monolayers from flasks inoculated with milk, which was spiked with virus and then pasteurised for 30 seconds at 72°C. Images (a), (b) and (c) represent samples spiked with 104 LSDV. Images (d), (e) and (f) represent samples spiked with 105 LSDV. Images (g), (h) and (i) represent samples spiked with 106 LSDV. Cell monolayers are intact and showing no signs of cytopathic effect (CPE) suggesting no viable LSDV is present in the samples.

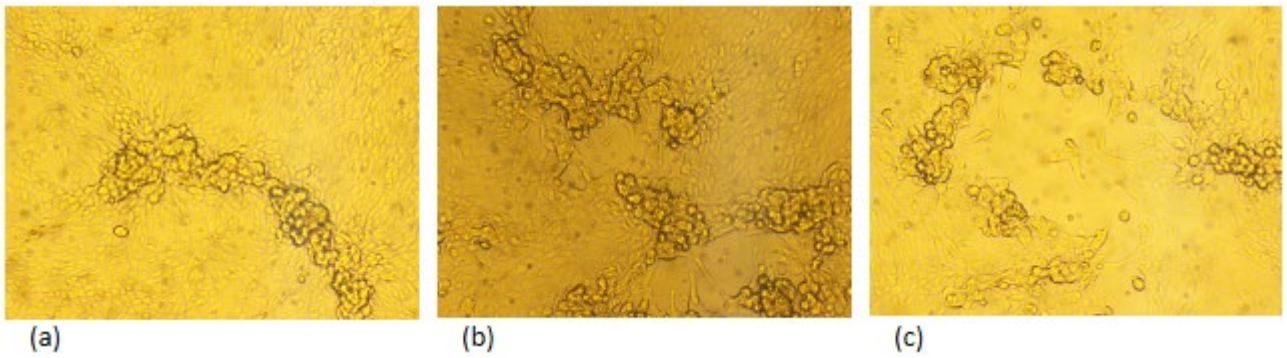


Figure 13. Cell monolayers infected with LSDV. Image (a) is inoculated with 10^4 LSDV, image (b) is inoculated with 10^5 LSDV and image (c) is inoculated with 10^6 LSDV. Typical LSDV-like CPE is observed across all three samples.

5.4 75°C for 7.5 seconds

Results from both $TCID_{50}$ and isolation demonstrated no viable virus remained in the milk samples that were pasteurised at 75°C for 7.5 seconds. No CPE was observed beyond toxicity in the $TCID_{50}$, and no virus like CPE was observed in the two passages of isolation on susceptible cells.

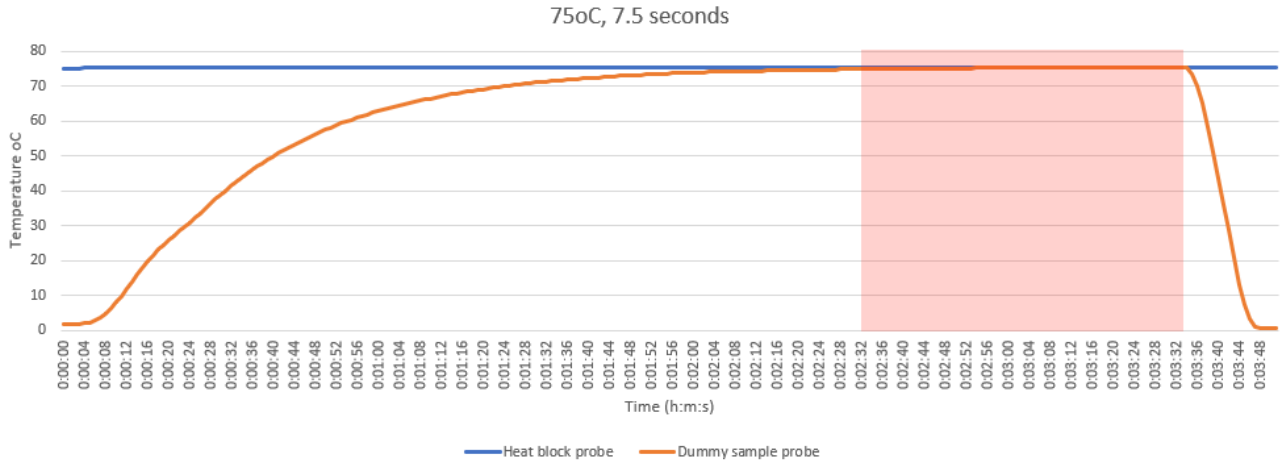


Figure 14. Temperature read out from the data logger files for the pasteurisation experiment. The blue line represents the temperature of the heat block, and the orange line represents the temperature of the dummy milk sample. The red box represents the period of time where the dummy sample reached the appropriate temperature, and spiking experiments were conducted in this time period.

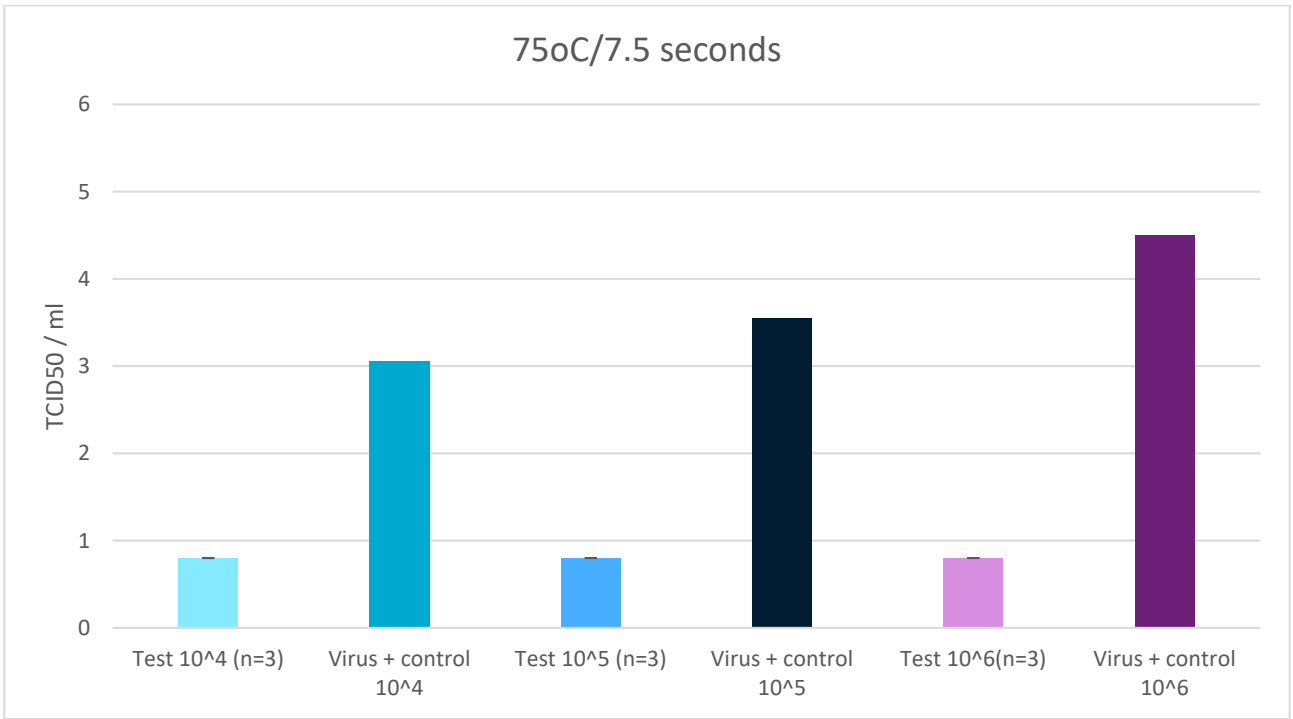


Figure 15. $TCID_{50}$ results for 75°C for 7.5 seconds – no infectious virus was detected in the 10^4, 10^5 and 10^6 samples (referred to as Test 10^4, Test 10^5 and Test 10^6, respectively). Positive controls (termed Virus + control) for each virus concentration (in milk, kept at 4°C) were performed alongside each test sample. The limit of detection for the assay is 0.8 $TCID_{50}$/mL.

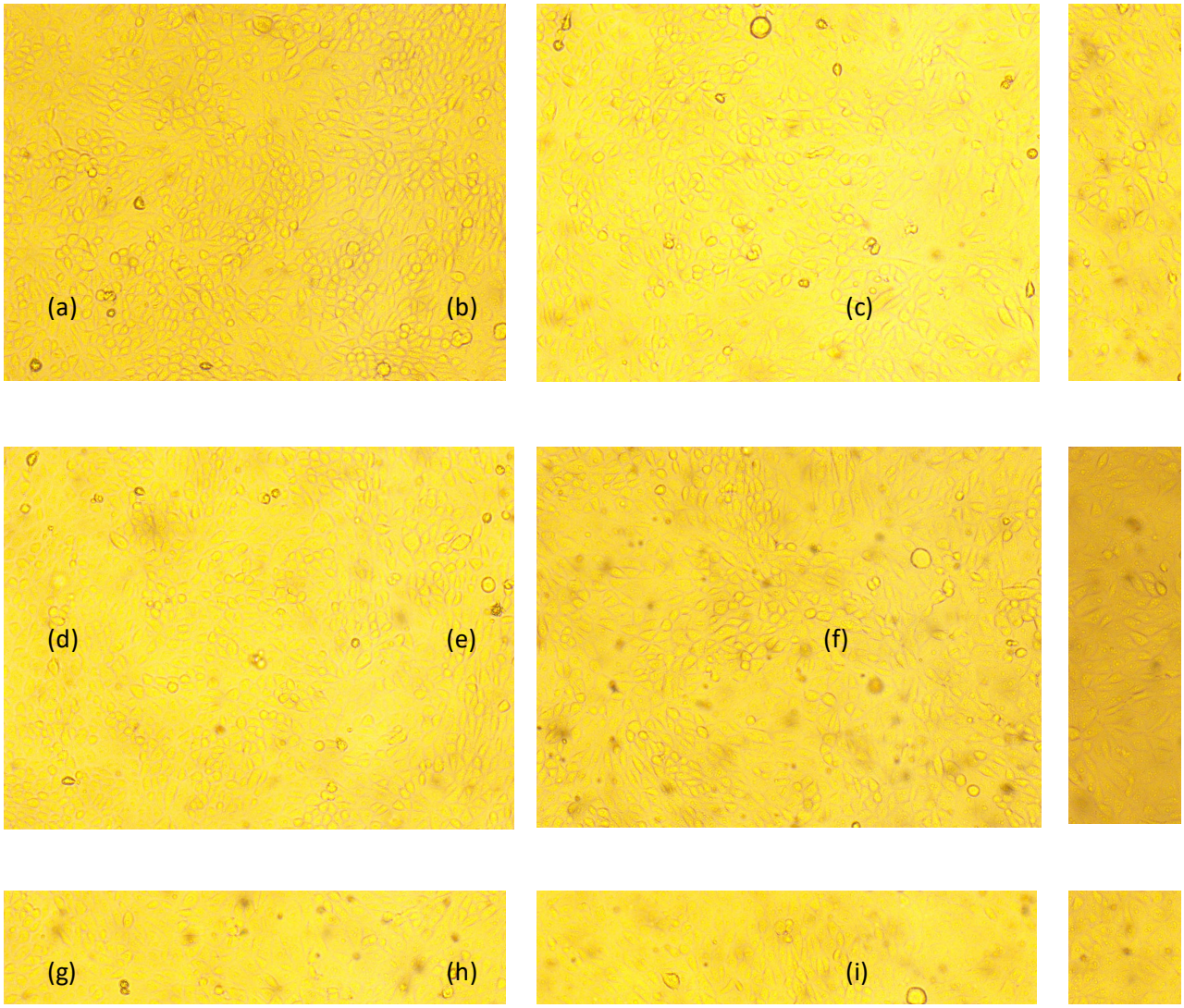


Figure 16. Cell monolayers from flasks inoculated with milk, which was spiked with virus and then pasteurised for 7.5 seconds at 75°C. Images (a), (b) and (c) represent samples spiked with 104 LSDV. Images (d), (e) and (f) represent samples spiked with 105 LSDV. Images (g), (h) and (i) represent samples spiked with 106 LSDV. Cell monolayers are intact and showing no signs of cytopathic effect (CPE) suggesting no viable LSDV is present in the samples.

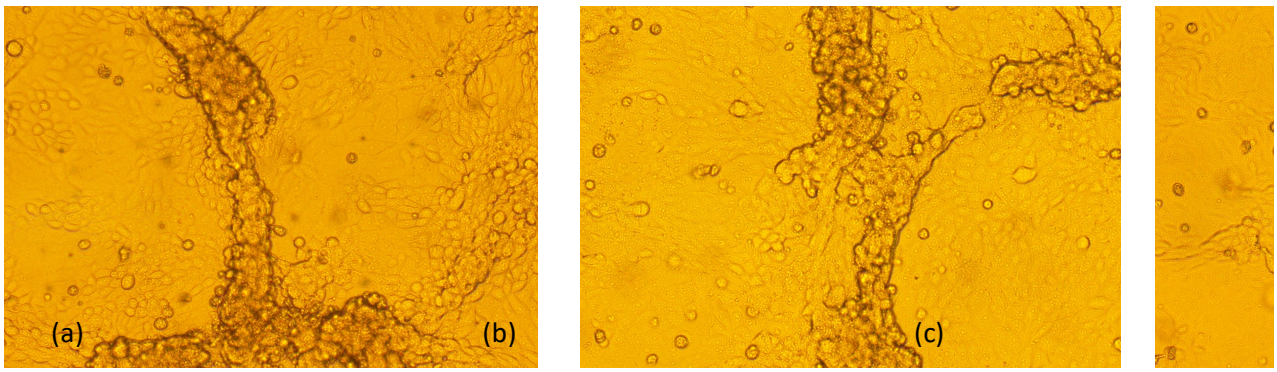


Figure 17. Cell monolayers infected with LSDV. Image (a) is inoculated with 10^4 LSDV, image (b) is inoculated with 10^5 LSDV and image (c) is inoculated with 10^6 LSDV. Typical LSDV-like CPE is observed across all three samples.

5.5 75°C for 15 seconds

Results from both $TCID_{50}$ and isolation demonstrated no viable virus remained in the milk samples that were pasteurised at 75°C for 15 seconds. No CPE was observed beyond toxicity in the $TCID_{50}$, and no virus like CPE was observed in the two passages of isolation on susceptible cells.

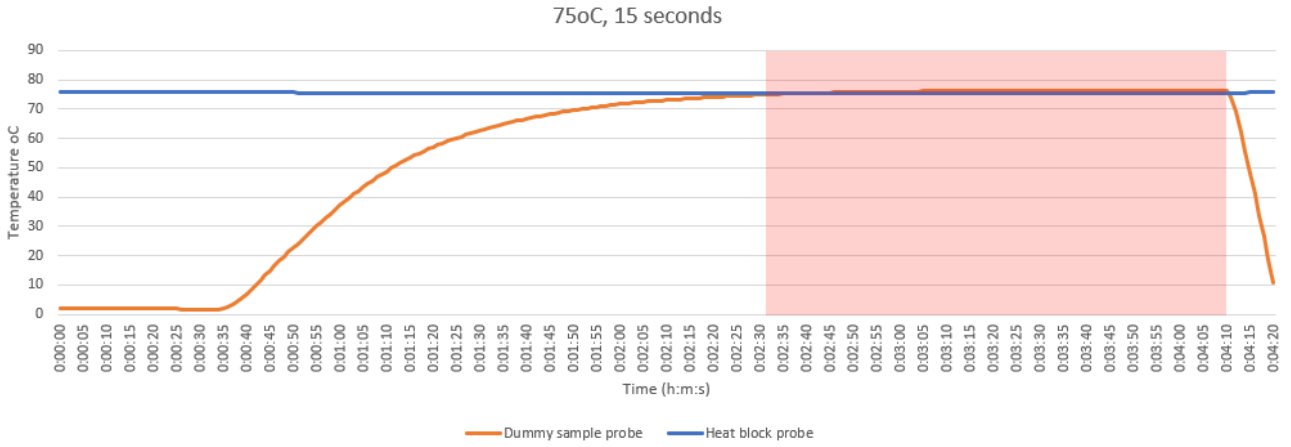


Figure 18. Temperature read out from the data logger files for the pasteurisation experiment. The blue line represents the temperature of the heat block, and the orange line represents the temperature of the dummy milk sample. The red box represents the period of time where the dummy sample reached the appropriate temperature, and spiking experiments were conducted in this time period.

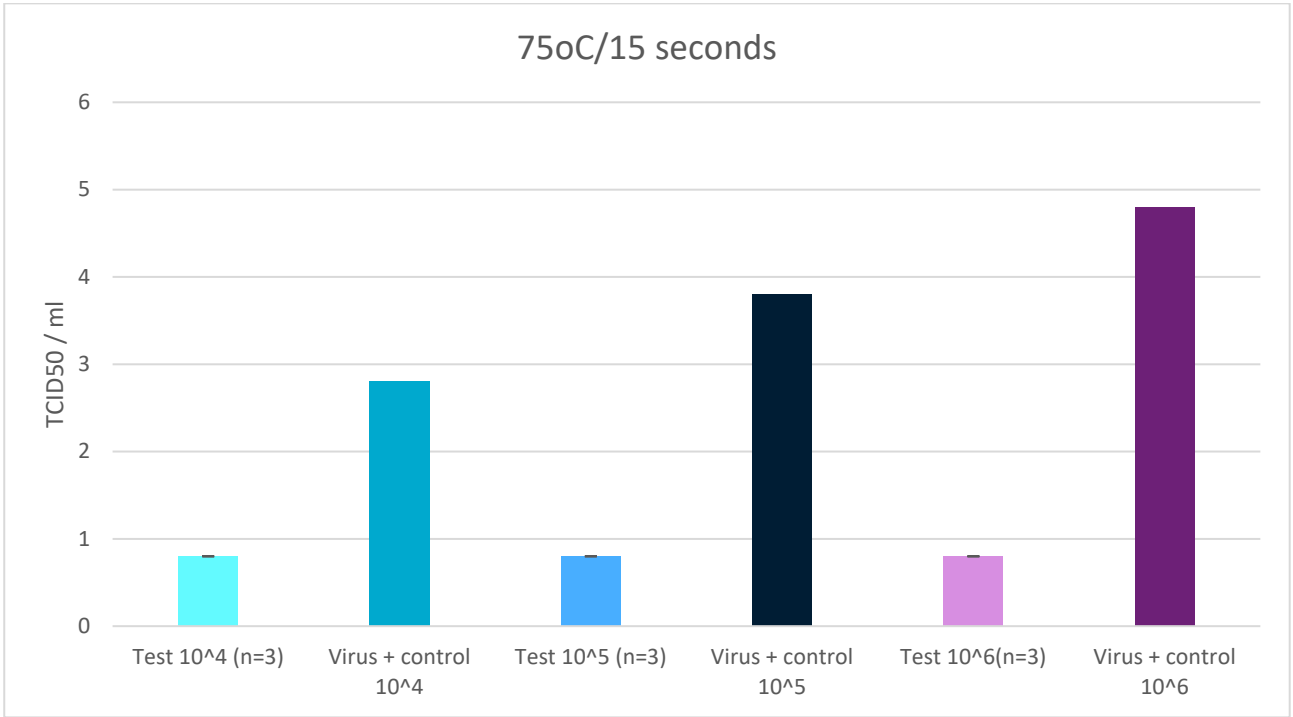


Figure 19. $TCID_{50}$ results for 75°C for 15 seconds – no infectious virus was detected in the 10^4, 10^5 and 10^6 samples (referred to as Test 10^4, Test 10^5 and Test 10^6, respectively). Positive controls (termed Virus + control) for each virus concentration (in milk, kept at 4°C) were performed alongside each test sample. The limit of detection for the assay is 0.8 $TCID_{50}$/mL.

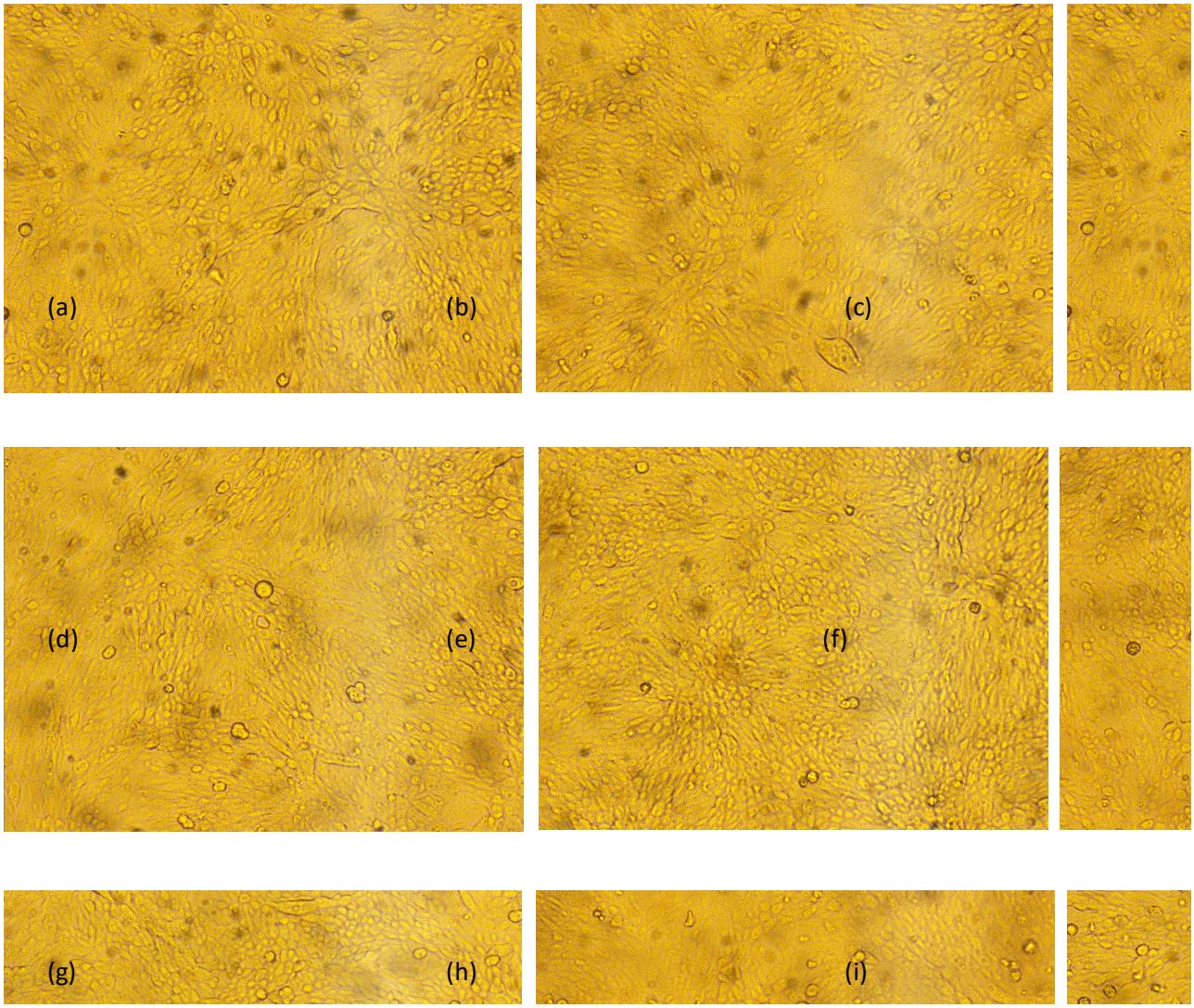


Figure 20. Cell monolayers from flasks inoculated with milk, which was spiked with virus and then pasteurised for 15 seconds at 75°C. Images (a), (b) and (c) represent samples spiked with 104 LSDV. Images (d), (e) and (f) represent samples spiked with 105 LSDV. Images (g), (h) and (i) represent samples spiked with 106 LSDV. Cell monolayers are intact and showing no signs of cytopathic effect (CPE) suggesting no viable LSDV is present in the samples.

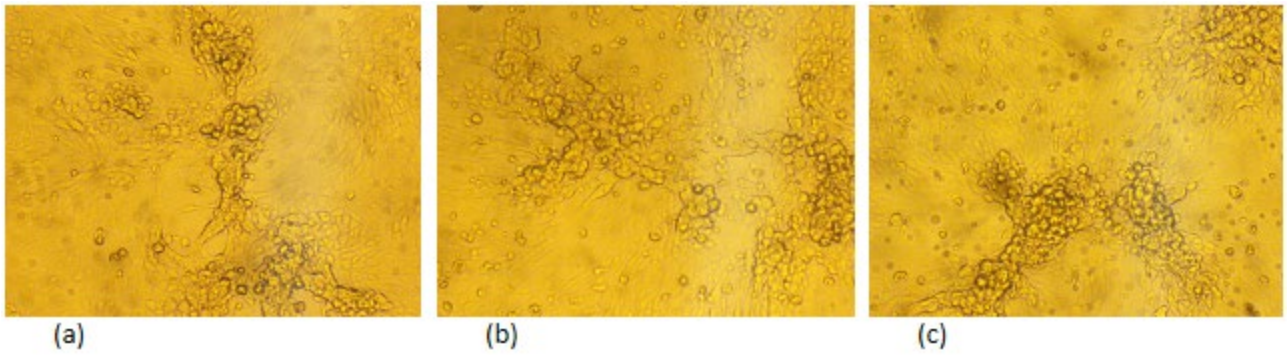


Figure 21. Cell monolayers infected with LSDV for the positive controls for the 75°C/15 second experiment. Image (a) is inoculated with 10^4 LSDV, image (b) is inoculated with 10^5 LSDV and image (c) is inoculated with 10^6 LSDV. Typical LSDV-like CPE is observed across all three samples.

5.6 75°C for 30 seconds

Results from both $TCID_{50}$ and isolation demonstrated no viable virus remained in the milk samples that were pasteurised at 75°C for 30 seconds. No CPE was observed beyond toxicity in the $TCID_{50}$, and no virus like CPE was observed in the two passages of isolation on susceptible cells.

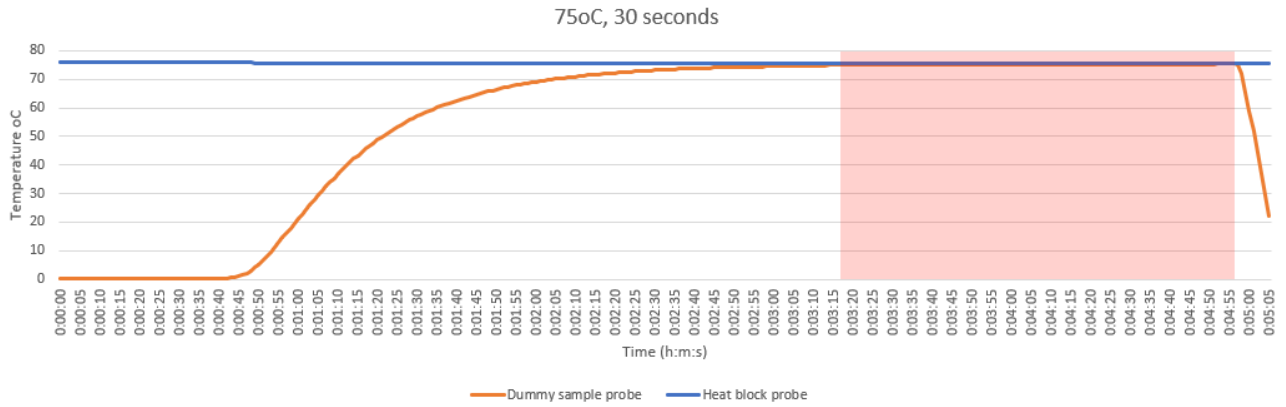


Figure 22. Temperature read out from the data logger files for the pasteurisation experiment. The blue line represents the temperature of the heat block, and the orange line represents the temperature of the dummy milk sample. The red box represents the period of time where the dummy sample reached the appropriate temperature, and spiking experiments were conducted in this time period.

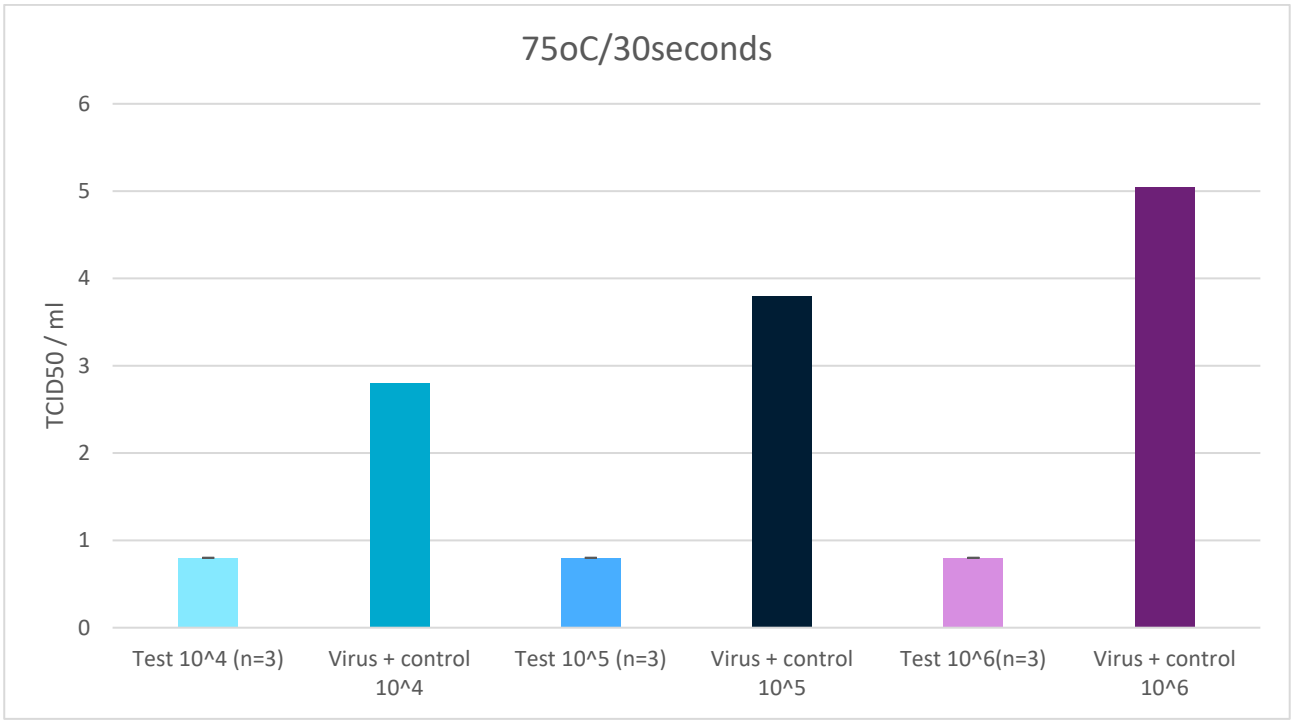


Figure 23. $TCID_{50}$ results for 75°C for 30 seconds – no infectious virus was detected in the 10^4, 10^5 and 10^6 samples (referred to as Test 10^4, Test 10^5 and Test 10^6, respectively). Positive controls (termed Virus + control) for each virus concentration (in milk, kept at 4°C) were performed alongside each test sample. The limit of detection for the assay is 0.8 $TCID_{50}$/mL.

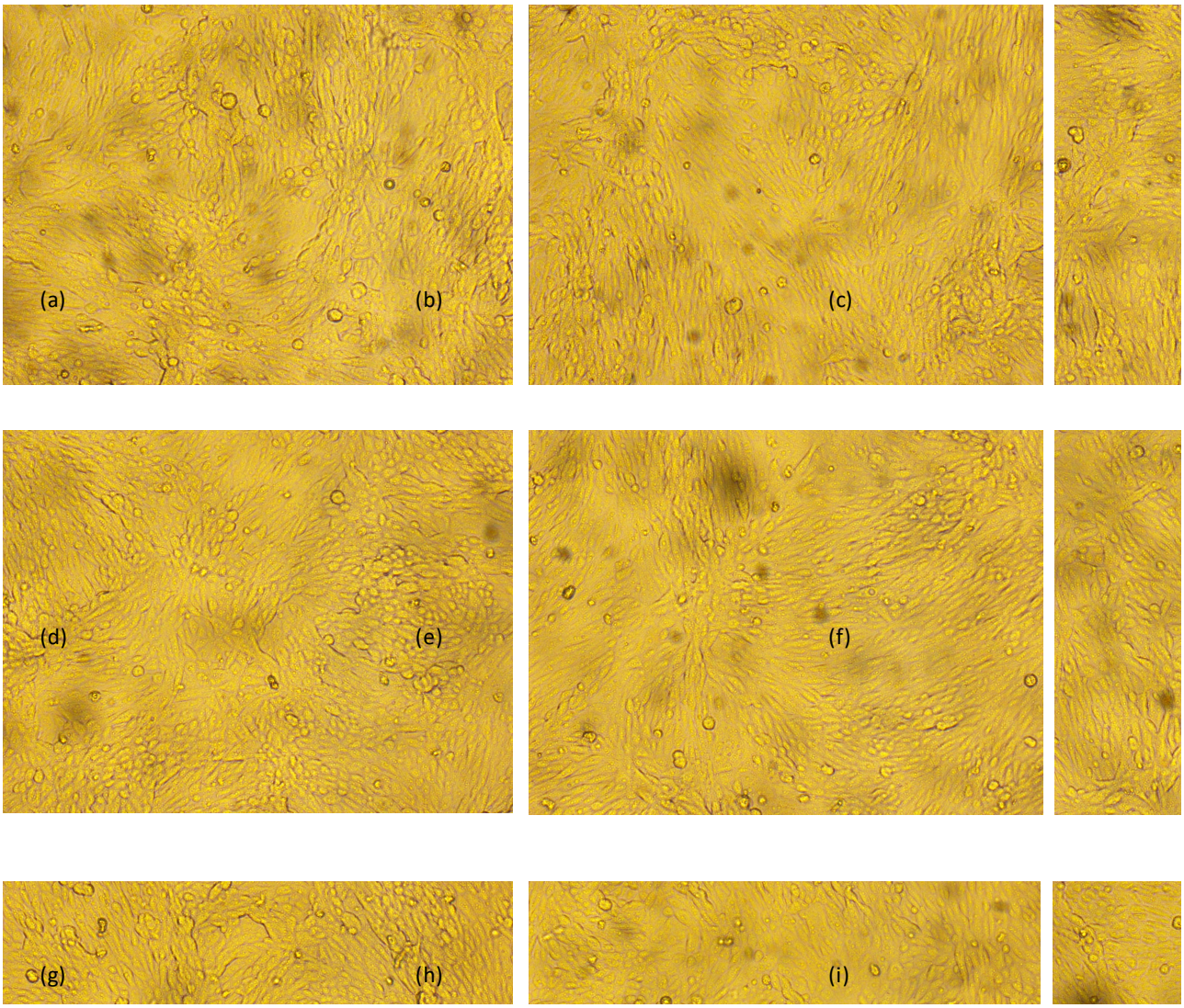


Figure 24. Cell monolayers from flasks inoculated with milk, which was spiked with virus and then pasteurised for 30 seconds at 75°C. Images (a), (b) and (c) represent samples spiked with 10^4 LSDV. Images (d), (e) and (f) represent samples spiked with 10^5 LSDV. Images (g), (h) and (i) represent samples spiked with 10^6 LSDV. Cell monolayers are intact and showing no signs of cytopathic effect (CPE) suggesting no viable LSDV is present in the samples.

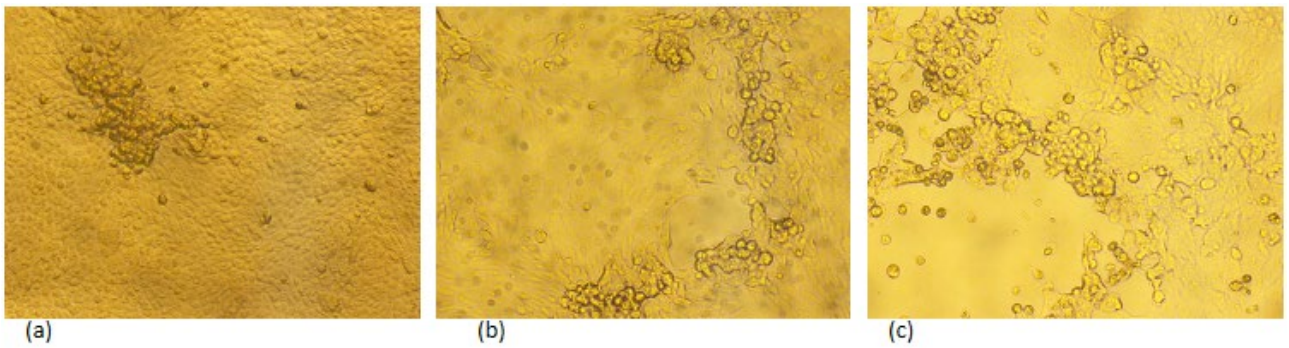


Figure 25. Cell monolayers infected with LSDV for the positive controls for the 75°C/30 second experiment. Image (a) is inoculated with 10^4 LSDV, image (b) is inoculated with 10^5 LSDV and image (c) is inoculated with 10^6 LSDV. Typical LSDV-like CPE is observed across all three samples.

5.7 80°C for 7.5 seconds

Results from both $TCID_{50}$ and isolation demonstrated no viable virus remained in the milk samples that were pasteurised at 80°C for 7.5 seconds. No CPE was observed beyond toxicity in the $TCID_{50}$, and no virus like CPE was observed in the two passages of isolation on susceptible cells.

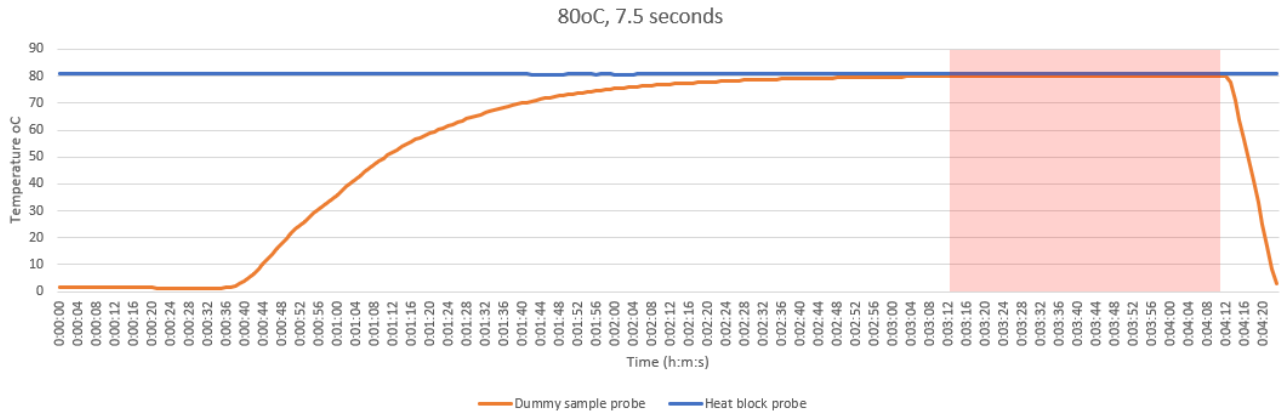


Figure 26. Temperature read out from the data logger files for the pasteurisation experiment. The blue line represents the temperature of the heat block, and the orange line represents the temperature of the dummy milk sample. The red box represents the period of time where the dummy sample reached the appropriate temperature, and spiking experiments were conducted in this time period.

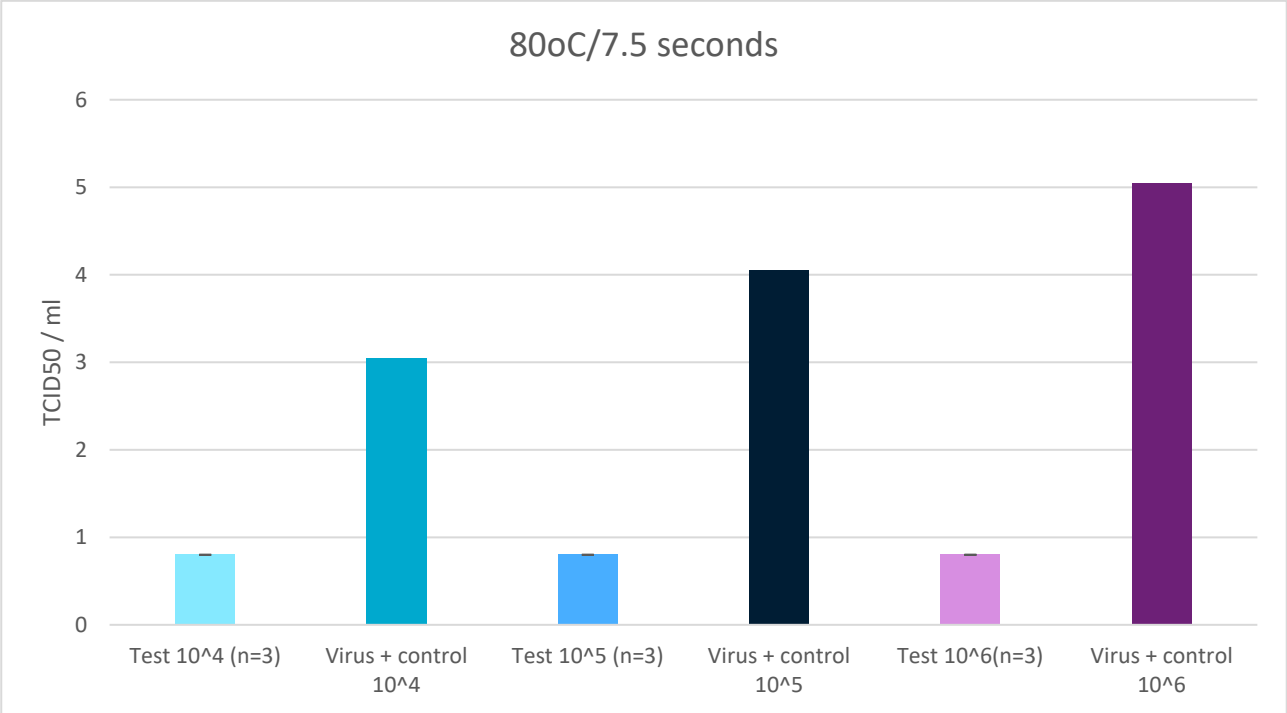


Figure 27. $TCID_{50}$ results for 80°C for 7.5 seconds – no infectious virus was detected in the 10^4, 10^5 and 10^6 samples (referred to as Test 10^4, Test 10^5 and Test 10^6, respectively). Positive controls (termed Virus + control) for each virus concentration (in milk, kept at 4°C) were performed alongside each test sample. The limit of detection for the assay is 0.8 $TCID_{50}$/mL.

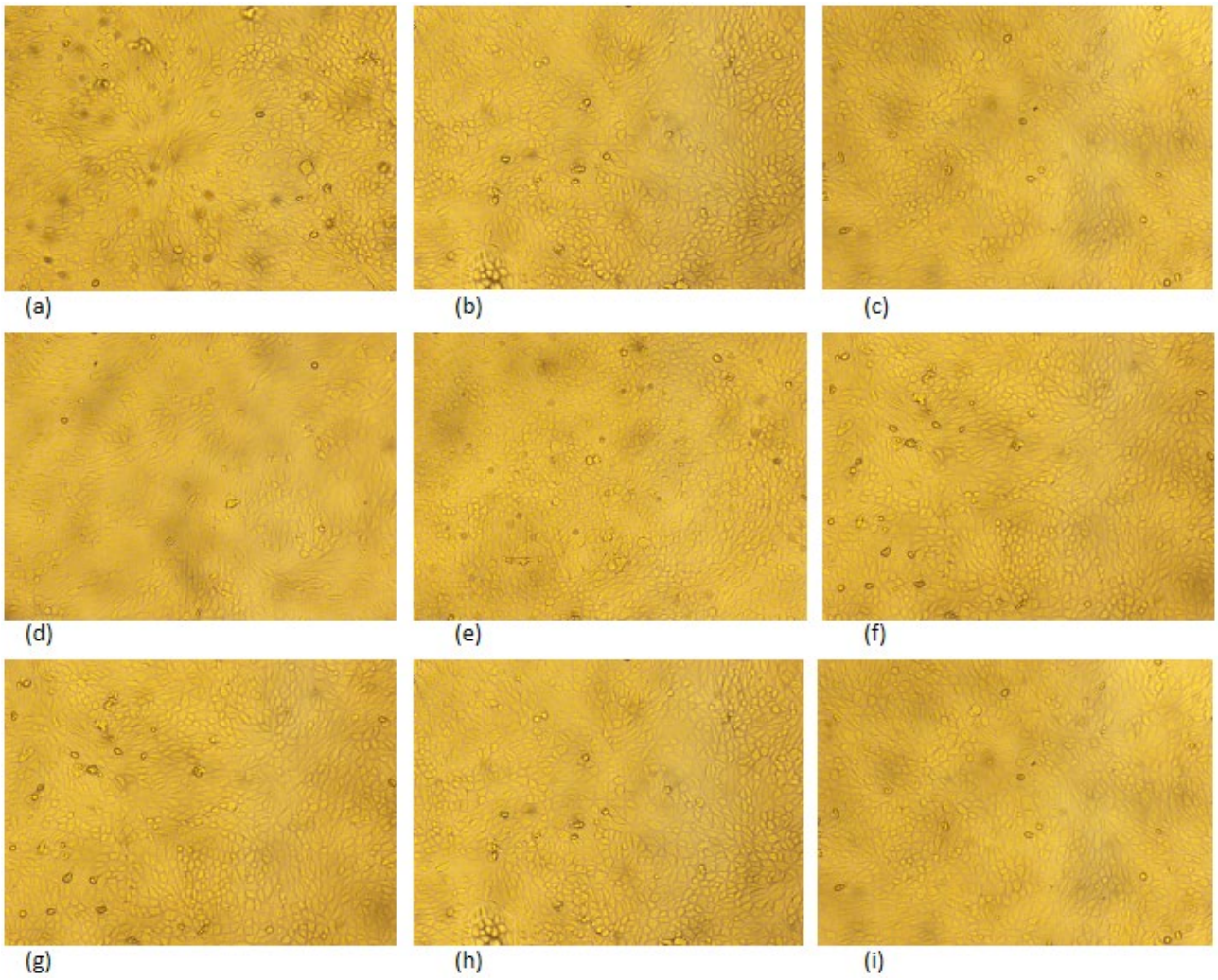


Figure 28. Cell monolayers from flasks inoculated with milk, which was spiked with virus and then pasteurised for 7.5 seconds at 80°C. Images (a), (b) and (c) represent samples spiked with 10^4 LSDV. Images (d), (e) and (f) represent samples spiked with 10^5 LSDV. Images (g), (h) and (i) represent samples spiked with 10^6 LSDV. Cell monolayers are intact and showing no signs of cytopathic effect (CPE) suggesting no viable LSDV is present in the samples.

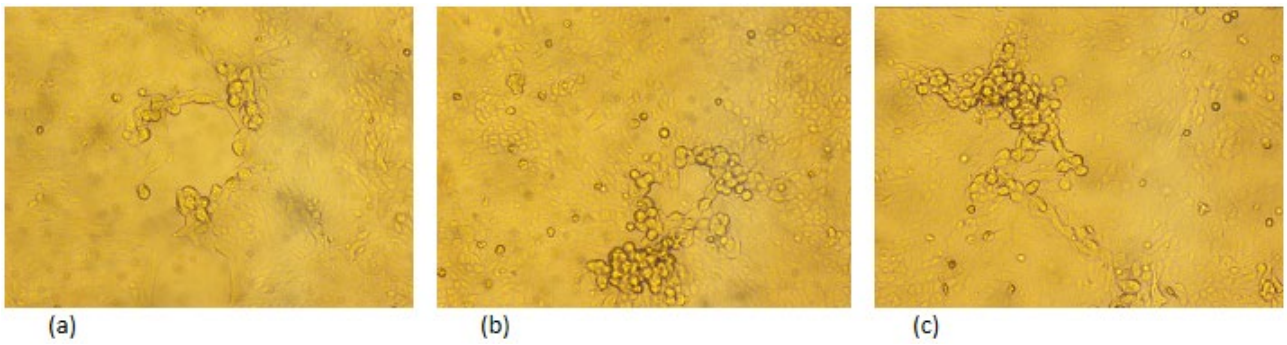


Figure 29. Cell monolayers infected with LSDV for the positive controls for the 80°C/7.5 second experiment. Image (a) is inoculated with 10^4 LSDV, image (b) is inoculated with 10^5 LSDV and image (c) is inoculated with 10^6 LSDV. Typical LSDV-like CPE is observed across all three samples.

5.8 80°C for 15 seconds

Results from both $TCID_{50}$ and isolation demonstrated no viable virus remained in the milk samples that were pasteurised at 80°C for 15 seconds. No CPE was observed beyond toxicity in the $TCID_{50}$, and no virus like CPE was observed in the two passages of isolation on susceptible cells.

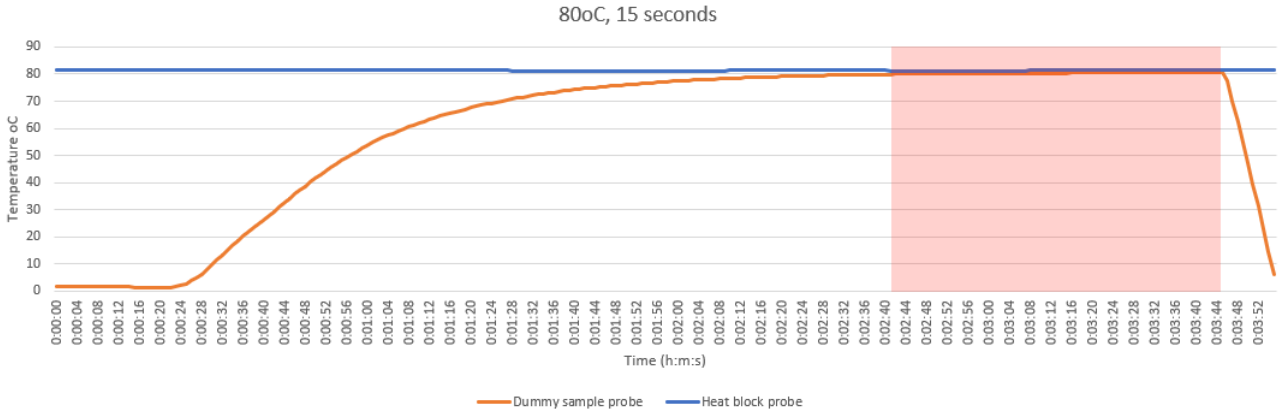


Figure 30. Temperature read out from the data logger files for the pasteurisation experiment. The blue line represents the temperature of the heat block, and the orange line represents the temperature of the dummy milk sample. The red box represents the period of time where the dummy sample reached the appropriate temperature, and spiking experiments were conducted in this time period.

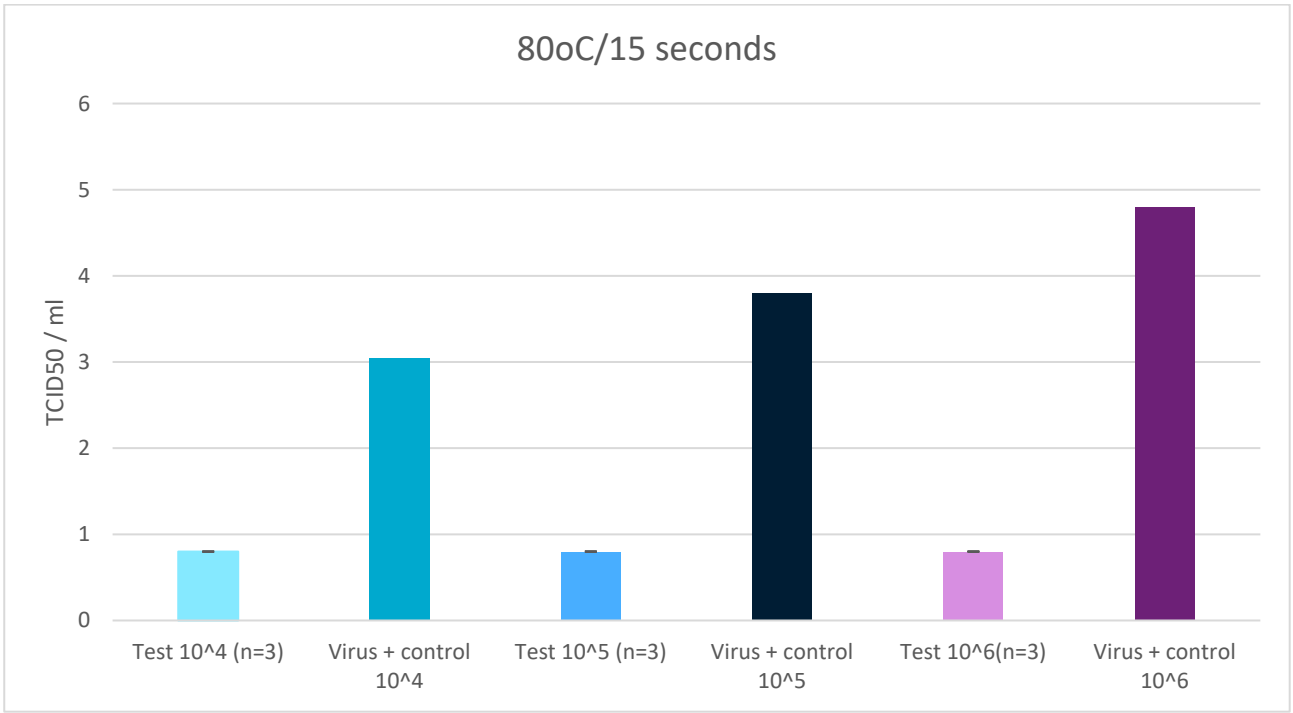


Figure 31. $TCID_{50}$ results for 80°C for 15 seconds – no infectious virus was detected in the 10^4, 10^5 and 10^6 samples (referred to as Test 10^4, Test 10^5 and Test 10^6, respectively). Positive controls (termed Virus + control) for each virus concentration (in milk, kept at 4°C) were performed alongside each test sample. The limit of detection for the assay is 0.8 $TCID_{50}$/mL.

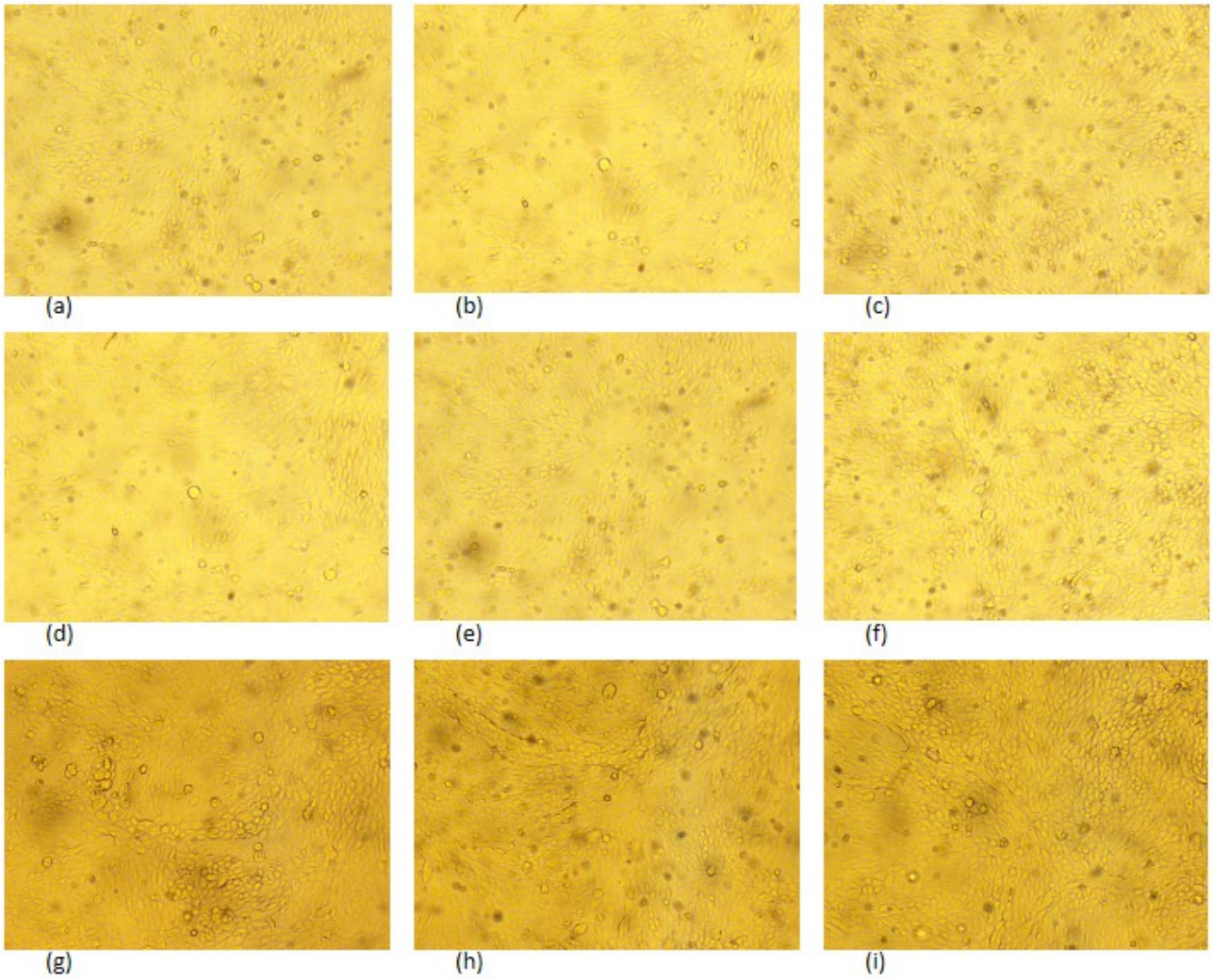


Figure 32. Cell monolayers from flasks inoculated with milk, which was spiked with virus and then pasteurised for 15 seconds at 80°C. Images (a), (b) and (c) represent samples spiked with 10^4 LSDV. Images (d), (e) and (f) represent samples spiked with 10^5 LSDV. Images (g), (h) and (i) represent samples spiked with 10^6 LSDV. Cell monolayers are intact and showing no signs of cytopathic effect (CPE) suggesting no viable LSDV is present in the samples.

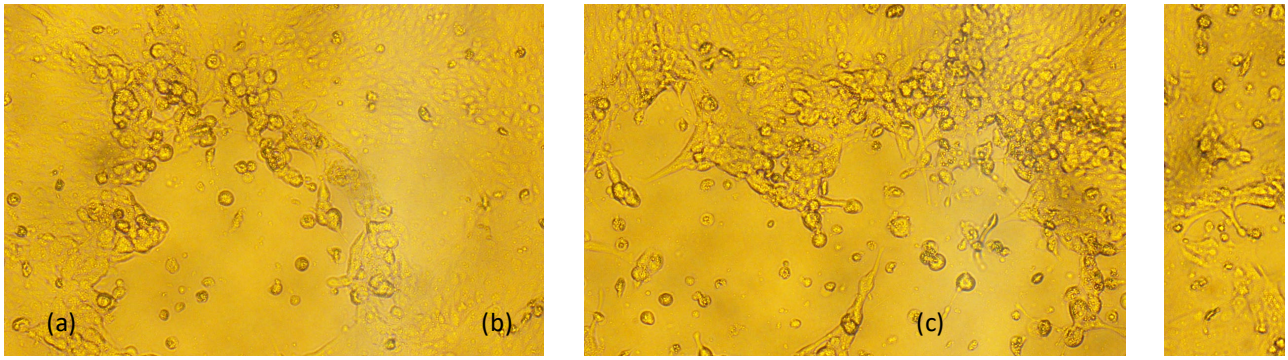


Figure 33. Cell monolayers infected with LSDV for the positive controls for the 80°C/15 second experiment. Image (a) is inoculated with 10^4 LSDV, image (b) is inoculated with 10^5 LSDV and image (c) is inoculated with 10^6 LSDV. Typical LSDV-like CPE is observed across all three samples.

5.9 80°C for 30 seconds

Results from both $TCID_{50}$ and isolation demonstrated no viable virus remained in the milk samples that were pasteurised at 80°C for 30 seconds. No CPE was observed beyond toxicity in the $TCID_{50}$, and no virus like CPE was observed in the two passages of isolation on susceptible cells.

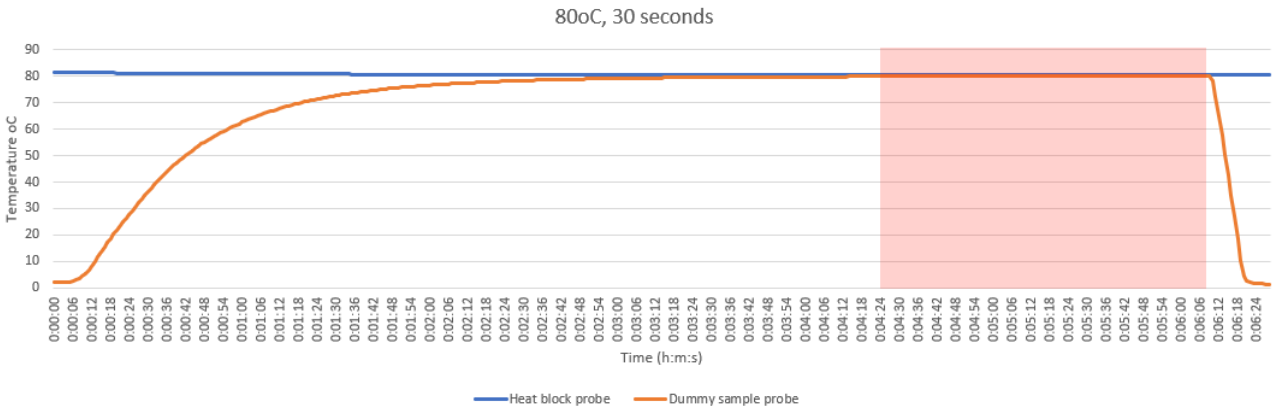


Figure 34. Temperature read out from the data logger files for the pasteurisation experiment. The blue line represents the temperature of the heat block, and the orange line represents the temperature of the dummy milk sample. The red box represents the period of time where the dummy sample reached the appropriate temperature, and spiking experiments were conducted in this time period.

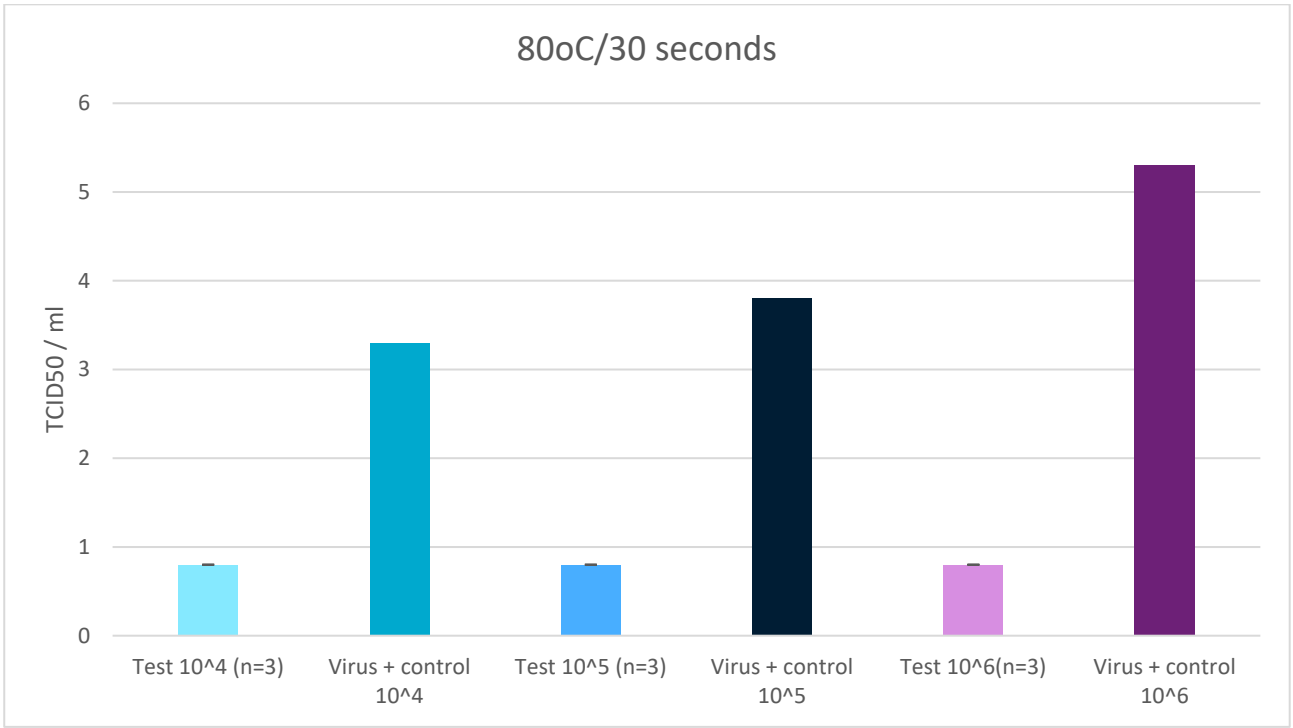


Figure 35. $TCID_{50}$ results for 80°C for 30 seconds – no infectious virus was detected in the 10^4, 10^5 and 10^6 samples (referred to as Test 10^4, Test 10^5 and Test 10^6, respectively). Positive controls (termed Virus + control) for each virus concentration (in milk, kept at 4°C) were performed alongside each test sample. The limit of detection for the assay is 0.8 $TCID_{50}$/mL.

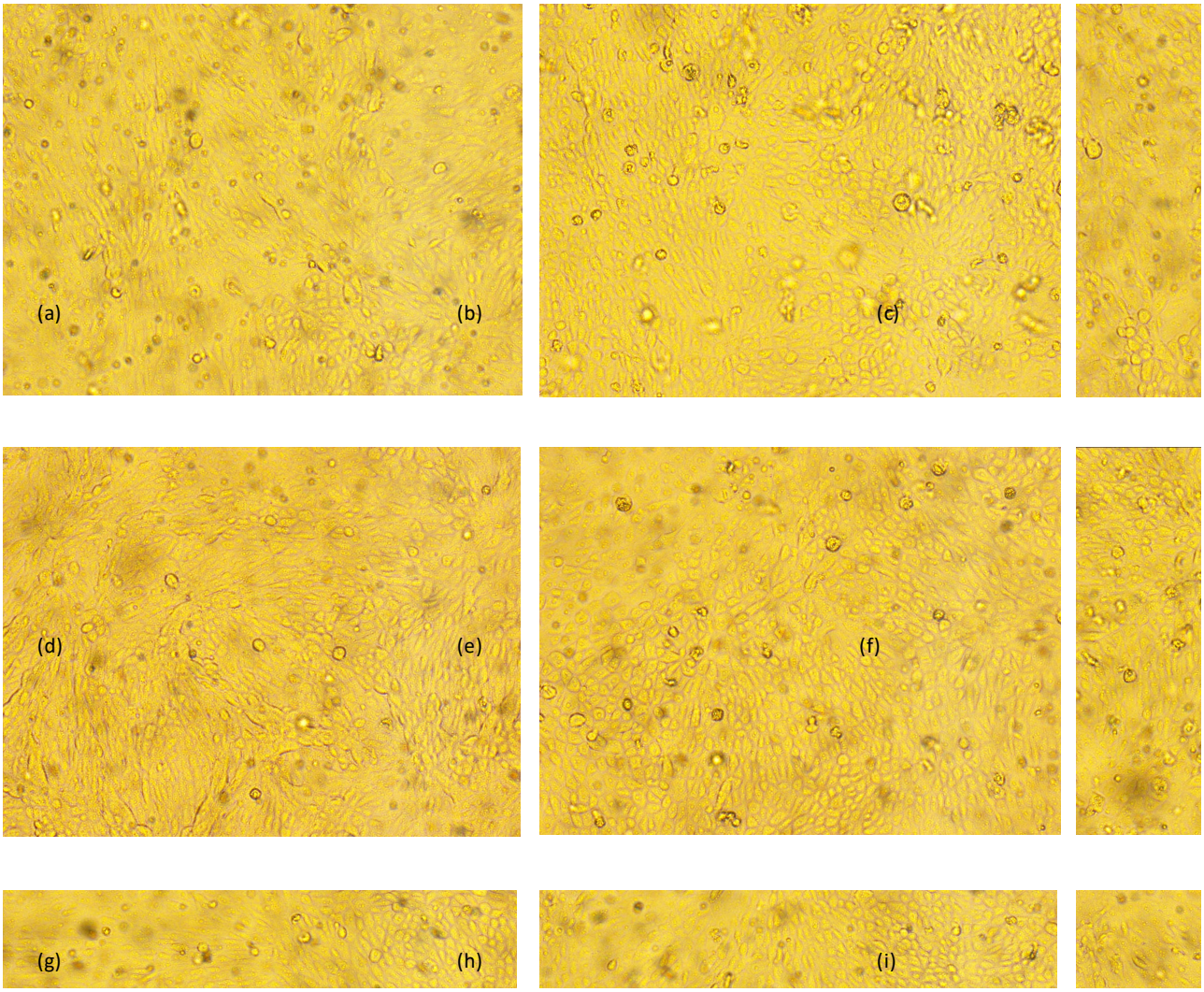


Figure 36. Cell monolayers from flasks inoculated with milk, which was spiked with virus and then pasteurised for 30 seconds at 80°C. Images (a), (b) and (c) represent samples spiked with 10^4 LSDV. Images (d), (e) and (f) represent samples spiked with 10^5 LSDV. Images (g), (h) and (i) represent samples spiked with 10^6 LSDV. Cell monolayers are intact and showing no signs of cytopathic effect (CPE) suggesting no viable LSDV is present in the samples.

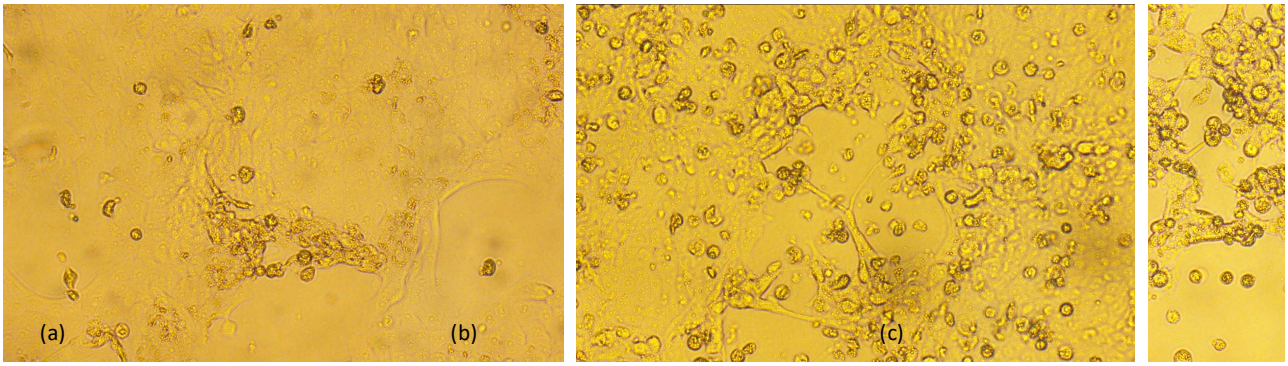


Figure 37. Cell monolayers infected with LSDV for the positive controls for the 80°C/30 second experiment. Image (a) is inoculated with 10^4 LSDV, image (b) is inoculated with 10^5 LSDV and image (c) is inoculated with 10^6 LSDV. Typical LSDV-like CPE is observed across all three samples.

6 Discussion

Results from initial testing to assess the suitability of milk in a cell culture detection system demonstrated that various components in milk are toxic in a $TCID_{50}$ system to a dilution of at least 1:100. To counter this the pasteurised milk was processed through Zeba Spin columns. Centrifuge columns such as the Zeba spin columns are routinely used for disinfectant testing as they allow for the removal of the toxic chemicals present in disinfectants to enable the resolution of virus viability more accurately. To further enhance detection of any possible infectious LSDV in the pasteurised milk samples, remaining milk sample from the Zeba spin columns, that was not used in the $TCID_{50}$ assay, was used for virus isolation on susceptible cells. The addition of isolation methodology provided greater sensitivity in detection of any remaining infectious virus particles.

Initial studies demonstrated that in a heat block, ice cold milk took a significant length of time to reach pasteurisation temperatures. A study on survival of Mycobacterium showed that the ramping time for raw milk in a water bath set to 72°C was approximately 3min 30 seconds, and that 2 minutes of this time was spent above 70°C (Gao et al 2002). This data supports our initial temperature trials where 250µL of milk took on average 2:34 seconds to reach 72°C. Additionally, it is a common laboratory method to inactivate virus at temperatures above 56°C, so any time spent above this temperature will cumulatively contribute to inactivation of LSDV. To reduce the chance of inactivation occurring during the long ramping period, and to match the conditions of the commercial setting more closely (approx. 10 second ramping time), it was decided to spike samples with virus once the milk had reached the set pasteurisation temperature. Once spiked, recording of the pasteurisation time commenced, and samples were chilled on ice immediately at the end of the set time period. This method ensured there was no effect of ramping up to temperature on the viability of the virus.

Results from the pasteurisation study demonstrated that no viable virus remained in milk treated at any combination of time, temperature or viral concentration tested in this study (7.5, 15, 30 seconds, 72°C, 75°C or 80°C or 10^4, 10^5 and 10^6 $TCID_{50}$/mL). Consistent recovery and titration of virus positive controls was observed across all viral titres tested and across experiments indicating reproducibility in results.

Results from this study correlate with previous pasteurisation studies on enveloped viruses. Complete inactivation of both Pseudorabies virus and Bovine Viral Disease Virus in human breast milk was observed in under 8 seconds at 72°C (Terpstra et al 2007).

7 Conclusion

Results from this study have demonstrated that any combination of time or temperature assessed in this study is sufficient to completely inactivate either 10^4, 10^5 or $10^6 TCID_{50}$/mL of LSDV in contaminated milk.

No detectable infectious virus was observed using pasteurisation parameters of 7.5 seconds at 72°C (half the time of Standard HTST pasteurisation temperatures utilised in Australia – 15 seconds at 72°C).

8 Glossary

BSCII	Biological Safety Cabinet Class II – standard protective equipment for infectious work – A BSCII provides both operator protection and protects the material from external contamination.
CPE	Cytopathic Effect – The change in morphology of a cell monolayer as a result of viral infection.
CSIRO	Commonwealth Scientific and Industrial Research Organisation.
DMEM	Dulbecco's Modified Eagle Media – the media used to grow the cellular detection system used for Lumpy Skin Disease Virus.
FCS	Foetal Calf Serum – a media supplement required for cellular growth and maintenance.
HTST	High Temperature Short Time – A pastuerisation method for dairy products, in Australia the parameters are 72°C for 15 seconds.
kDa	Kilodalton – an atomic mass unit of molecule – typically used for protein sizes, in this instance refers to the molecular cut off weight of the Zeba spin columns.
LSDV	Lumpy Skin Disease Virus.
MDBK	Madin-Darby Bovine Kidney cells – the cell type used to isolate and amplify Lumpy Skin Disease Virus.
mL	Millilitre – a unit of capacity equal to one thousandth of a litre.
STE	Sodium, TRIS, EDTA buffer – a standard laboratory buffer used to resuspend virus following centrifugation.
$TCID_{50}$	Tissue Culture Infectious Dose 50 – the minimum number of virus particles to cause infection 50% of the time. For example, a $TCID_{50}$ of $1x10^3$ equals 1000 times the minimum number of virus particles required to cause infection in 50% of samples.
µL	Microlitre- $1/1000^{th}$ of a Millilitre.

9 References

Terpstra FG, Rechtman DJ, Lee ML, van Hoeij K, Berg H, Van Engelenburg FAC, Van 't Wout AB (2007) Antimicrobial and Antiviral Effect of High-Temperature Short-Time (HTST) Pasteurization Applied to Human Milk, Breastfeeding Medicine, 2(1)

Gao A, Mutharia L, Chen S, Rahn, K, Odumeru J (2002) Effect of Pasteurization on Survival of Mycobacterium paratuberculosis in Milk, J. Dairy Sci. 85:3198-3205

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