Thermal inactivation spectrum of influenza A H5N1 virus in raw milk

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Abstract

The spillover of highly pathogenic avian influenza (HPAI) H5N1 virus to dairy cows and shedding of high amounts of infectious virus in milk raised public health concerns. Here, we evaluated the decay and thermal stability spectrum of HPAI H5N1 virus in raw milk. For the decay studies, HPAI H5N1 positive raw milk was incubated at different temperatures and viral titers and the thermal death time D-values were estimated. We then heat treated HPAI H5N1 virus positive milk following different thermal conditions including pasteurization and thermization conditions. Efficient inactivation of the virus was observed in all tested conditions, except for thermization at 50°C 10 min. Utilizing a submerged coil system with temperature ramp up times that resemble commercial pasteurizers, we showed that the virus was rapidly inactivated by pasteurization and most thermization conditions. These results provide important insights on the food safety measures utilized in the dairy industry.

Introduction

Highly pathogenic avian influenza (HPAI) H5N1 virus clade 2.3.4.4b was first detected in the United States on February 8, 2022[1]. Since then, the virus has been detected in over 1,170 poultry flocks in 48 states resulting in the death or culling of more than 100 million birds[1]. A unique feature observed throughout the HPAI H5N1 panzootic, is the frequent spillover of the virus to wild terrestrial and aquatic mammalian species with at least 24 species confirmed positive to date[2,3], including important livestock species such as goats and dairy cows [4–6]. Spillover of HPAI H5N1 virus to dairy cows and detection of high infectious viral loads and shedding in milk from infected cows[6,7] raised public health concerns given the potential for zoonotic spillover of the virus. As of September 10, 2024, there have been 15 confirmed human cases in the USA since the beginning of the HPAI H5N1 panzootic in 2022[8], 14 of those documented since the reported spillover of the virus to dairy cows in March 2024. Importantly, four of the 2024 human cases have been linked to occupational exposures to dairy cows, nine were linked to exposures to poultry, while, the most recent case reported by CDC on September 6, 2024, in Missouri - a state where the virus has not yet been detected in dairy cows – is the first human case without a known occupational exposure to infected animals[8].

Thermal treatment of milk or pasteurization is the main procedure used by the dairy industry to ensure safety of dairy products and extend their shelf-life by reducing and destroying non-pathogenic and pathogenic microorganisms[9]. Currently, in the USA two pasteurization conditions are approved by the U.S. Food and Drug Administration (FDA) for milk including low temperature long time (LTLT) treatment at or above 63°C for 30 min and high temperature short time (HTST) treatment at or above 72°C for 15 sec. Early studies reporting the detection of HPAI H5N1 viral RNA in retail milk and other dairy products, such as cheese, butter and ice cream[10,11], coupled with laboratory studies suggesting that heat treatment of raw HPAI H5N1 positive milk at 72°C for 15 sec, the most common pasteurization method used in the USA, did not completely inactivate the virus[12] heightened public health concerns about the potential risk of human infections through milk. Confirmation that no infectious virus was present in retail milk products subjected to pasteurization eased some of the initial public health concerns[11]. However, the public health risk posed by consumption of raw milk or milk subjected to subpasteurization conditions, a process known as thermization which is milder than pasteurization and involves heating to a

temperature typically between 50-68ºC for about 15 to 30 sec to up to 10 min, remains unknown. Notably, while thermization reduces the number of non-sporeforming pathogens and psychrotrophic spoilage bacteria, it does not destroy all pathogenic microorganisms present in milk[13,14].

Here, we investigated the decay of HPAI H5N1 virus in milk and evaluated the efficacy of different heat treatment conditions including the FDA approved pasteurization conditions and several thermization conditions on inactivation of HPAI H5N1. The work provides a comprehensive overview of the thermal inactivation spectrum of HPAI H5N1 virus in raw milk.

Results

Highly pathogenic avian influenza (HPAI) H5N1 virus presents long term stability in raw milk at 4ºC but is rapidly inactivated at higher temperatures. We studied the decay of HPAI H5N1 virus in raw milk at different temperatures. For this, we incubated HPAI H5N1 virus positive raw milk collected from infected cows or normal raw milk spiked with HPAI H5N1 virus at 4°C, 20°C, 30°C and 37°C and collected sequential samples daily between days 1-7 and weekly thereafter until day 56 (8 weeks). HPAI H5N1 virus decay over time was assessed by viral titrations performed in bovine uterine epithelial cells (Cal-1). Samples that were toxic or negative in the cell culture titration assay were subjected to confirmatory virus isolation in embryonated chicken eggs (ECEs). Virus titers were used to estimate the thermal death time values (D-values) in each studied temperature. At 4°C, there was about 4 log reduction in infectious virus titers from 5.97 ± 0.14 log $TCID_{50}$/mL on day 0 to 2.05 ± 0.43 log $TCID_{50}$/mL on day 42 **(Fig. 1a)**. Samples from days 49 and 56 were toxic to cell culture, but when inoculated in ECEs both samples were confirmed to contain infectious HPAI H5N1 virus (**Extended Data Table 1**), demonstrating the long-term stability of the virus in raw milk stored 4°C. The D-value of HPAI at 4°C was estimated to be 10.74 days.

At 20°C, we observed 3 log reduction in infectious virus titers within seven days, with titers dropping from 6.88 ± 0.14 log $TCID_{50}$/mL on day 0 to 3.88 ± 0.14 log $TCID_{50}$/mL on day 7 **(Fig. 1b)**. Samples collected on days 14 and onward were toxic to cell culture and, thus, were subjected to inoculation in ECEs. One out of three milk samples collected on day 14 led to embryo mortality, which was confirmed to be caused by HPAI H5N1 virus by hemagglutination (HA) assay (**Extended Data Table 1**). No embryo mortality was observed in milk samples collected on

112 day 21 and the allantoic fluids collected from inoculated ECEs were HA negative, indicating virus
113 inactivation in milk stored at 20°C at day 21 (**Extended Data Table 1**). The estimated D-value of
114 HPAI H5N1 virus in milk stored at 20°C was 2.23 days.

115 At 30°C, we detected a rapid decrease in infectious HPAI H5N1 virus titers in milk, which
116 dropped from 5.88 ± 0.14 log $TCID_{50}$/mL on day 0 to 1.63 ± 0.29 log $TCID_{50}$/mL on day 5 resulting
117 in about 4-log reduction in infectious virus titers **(Fig. 1c)**. No infectious virus was detected in
118 milk samples from day 6 and onward when we used both cell culture and ECEs to assess virus
119 infectivity **(Fig. 1c, Extended Data Table 1)**. The estimated D-value of HPAI H5N1 virus in milk
120 stored at 30°C was 1.16 days.

121 At 37°C, we observed a drastic drop in HPAI H5N1 virus titers, with a 3-log reduction in
122 virus infectivity within the first day of incubation, decreasing from 5.88 ± 0.14 log $TCID_{50}$/mL on
123 day 0 to 2.88 ± 0.14 log $TCID_{50}$/mL on day 1 **(Fig. 1d)**. From day 2 and onwards, no infectious
124 virus was detected when samples were inoculated in cell culture and ECEs **(Fig. 1d, Extended**
125 **Data Table 1)**. The estimated D-value of HPAI H5N1 virus in milk stored at 37°C was 0.34 days.
126 Together these results show that HPAI H5N1 virus presents long term stability in raw milk stored
127 at 4°C, with viral titers decreasing rapidly at temperatures above 30°C.

128 **Thermal treatment of raw HPAI H5N1 virus positive milk following thermization conditions**
129 **and FDA approved pasteurization results in efficient virus inactivation.** Initially, we evaluated
130 the efficacy of thermization at 60°C for 10 min and the FDA approved pasteurization conditions
131 (63°C for 30 min and 72°C for 15 sec), on the inactivation of HPAI H5N1 virus in raw milk
132 collected from clinically affected cows or in raw milk spiked with HPAI H5N1 virus utilizing a
133 thermocycler. Following heat treatment, milk samples were subjected to virus inoculation and
134 titrations in Cal-1 cells and ECEs. While robust virus replication was detected by
135 immunofluorescence (IFA) in cells inoculated with control non-heat-treated milk samples, no
136 evidence of virus infectivity in raw milk subjected to heat treatment at 60°C for 10 min and at
137 63°C for 30 min or 72°C for 15 sec was observed in Cal-1 cells (**Fig. 2a**).

138 We then quantified viral RNA by qRT-PCR and determined infectious virus titers in control
139 and heat-treated milk samples using cell culture and ECEs. Similar levels of viral RNA were
140 detected in control and in heat-treated milk samples (**Fig. 2b**). Importantly, viral titrations revealed
141 that non-heat-treated raw milk clinical samples had a virus titer of 6.47 ± 0.29 log $TCID_{50}$/mL and

142 7.47 ± 0.29 log EID_{50}/mL, while no infectious virus was detected after heat treatment of the raw
143 milk clinical samples, in cell culture or ECEs, respectively (**Fig. 2c-2d**).

144 We further assessed the thermal inactivation of HPAI H5N1 in normal raw milk spiked
145 (1:10 ratio) with raw milk positive for HPAI H5N1 collected from clinically affected cows. We
146 followed the same heat treatment conditions described above utilizing a thermocycler. Similar to
147 the results observed in raw milk from clinical cows, robust HPAI H5N1 virus replication was
148 observed in Cal-1 cells inoculated with non-heat-treated spiked milk samples, whereas no virus
149 replication was observed after heat treatment at 60°C for 10 min or at 63°C for 30 min or 72°C for
150 15 sec (**Fig. 2e**). While the viral RNA levels remained stable after heat treatment (**Fig. 2f**),
151 complete virus inactivation was observed in spiked milk samples following heat treatment (**Fig.
152 2g-2h**). These results suggest that thermization at 60°C for 10 min and pasteurization at 63°C for
153 30 min or 72°C for 15 sec efficiently led to HPAI H5N1 virus inactivation in raw milk.

154 **Evaluation of thermization conditions on HPAI H5N1 virus inactivation in raw milk.** We next
155 evaluated the efficacy of three additional thermization conditions on HPAI H5N1 virus inactivation
156 in raw milk. For this, raw normal milk was spiked with a HPAI H5N1 virus stock amplified in Cal-
157 1 cells (1:20 ratio) and subjected to thermal treatment at 50°C for 10 min, 63°C for 22 sec and
158 69°C for 22 sec utilizing a thermocycler. Inoculation of the control and heat-treated samples in
159 Cal-1 cells revealed robust HPAI H5N1 virus replication in control non-treated raw spiked milk
160 samples. Additionally, HPAI H5N1 virus replication was detected by IFA in cells inoculated with
161 raw spiked milk heat treated at 50°C for 10 min, however, the proportion of infected cells and the
162 intensity of staining was lower compared to the control non-treated raw milk samples (**Fig. 3a**).
163 No virus replication was detected in cells inoculated with raw HPAI-spiked milk samples heated
164 at 63°C for 22 sec and 69°C for 22 sec, suggesting efficient virus inactivation under these thermal
165 conditions.

166 Quantification of HPAI H5N1 virus RNA by qRT-PCR revealed similar viral RNA levels
167 in control non-treated and all three heat-treated raw spiked milk samples (**Fig. 3b**). Viral titrations
168 have shown that heat treatment of raw HPAI-spiked milk at 50°C for 10 min led to 1.5-2 log
169 reduction in infectious virus titers, which decreased from 4.94 ± 0.21 to 3.19 ± 0.81 log $TCID_{50}$/mL
170 or 4.87 ± 0.13 to 2.87 ± 0.35 log EID_{50}/mL after heat treatment (**Fig. 3c-d**). No infectious virus
171 was detected in raw HPAI-spiked milk samples heat treated at 63°C for 22 sec and 69°C for 22

172 sec. These results show that while thermization at 50°C for 10 min does not completely inactivate
173 HPAI H5N1 virus in raw milk, higher temperatures of 63 and 69°C efficiently and rapidly
174 inactivate the virus in raw milk.

175 **Validation of thermal inactivation of HPAI H5N1 virus in raw milk using a submerged coil**
176 **system.** To more closely simulate the thermal treatment conditions (e.g. circulating hot water heat
177 source and rapid ramp up temperatures) to which raw fluid milk is subjected to during commercial
178 pasteurization in the dairy industry, we developed a submerged coil heating system to heat treat
179 milk samples (**Fig. 4a**). The system holds 15 mL of milk within a coil and is heated with a
180 circulating water bath. The submerged coil heating system is furnished with four thermal sensors
181 that are connected to a data logger to continuously monitor and record the milk temperature as it
182 flows through four segments of the coil (**Fig. 4a**). The first coil segment (capacity of 4.2 mL)
183 contains two portions, including one external coil component carrying the sample injection port
184 and a temperature sensor (T1) (**Fig. 4a**) to monitor the temperature of the milk at the injection port
185 (**Fig. 4b**); and one submerged coil component (**Fig. 4a**) which preheats the milk sample to the
186 desired temperature within 7-10 sec (**Fig. 4b**) as it flows through this portion of the coil. The
187 temperature of the milk that flows through the pre-heating coil segment is recorded by thermal
188 sensor T2 (**Fig. 4a** and **4b**). The second (capacity 5.5 mL) and third (capacity 5.7 mL) coil
189 segments are completely submerged in water and hold the sample at the target temperature for the
190 intended time. The thermal sensor T3 monitors the milk temperature in the holding coil segment
191 (**Fig. 4a** and **4b**). The fourth coil segment (capacity of 1.5 mL) consists of the exit loop which
192 dispenses the milk in a collection tube after the heat treatment. Cooling of the milk after thermal
193 treatment was achieved by placing the collection tubes on ice for 10 min, immediately after sample
194 collection.

195 To validate the results obtained with the thermocycler, we evaluated the thermal
196 inactivation of HPAI in raw milk using the submerged coil system following the FDA approved
197 pasteurization conditions at 63°C for 30 min and 72°C for 15 s, and four thermization conditions
198 at 50°C for 10 min, 60°C for 10 min, 63°C for 22s and 69°C for 22s. For each thermal treatment
199 condition, 15 mL of raw milk spiked with HPAI H5N1 virus (1:20 ratio) were injected into the
200 submerged coil heating system and subjected to heat treatment at the desired temperatures and
201 times. Temperature monitoring as recorded by the thermal sensors data loggers following each

202 treatment confirmed that the milk samples were subjected to the heat treatment at the target
203 temperatures (**Fig. 4b, Extended Data Fig. 1**). Initially, virus infectivity was monitored by
204 inoculation of control and thermal treated spiked milk samples in Cal-1 cells. As shown in **Fig. 4c**,
205 virus replication was detected in control non-treated samples and in milk samples subjected to heat
206 treatment at 50°C for 10 min, as evidenced by positive viral NP staining in inoculated cells. Similar
207 to the results obtained with the experiments conducted in the thermocycler, a lower number of NP
208 positive cells were observed in cells inoculated with milk samples subjected to heat treatment at
209 50°C for 10 min, when compared to control non-treated samples (**Fig. 4c**). No evidence of virus
210 replication was detected in HPAI-spiked milk samples heat treated at 60°C for 10 min, 63°C for
211 22 s, 63°C for 30 min, 69°C for 22 s and 72°C for 15 s.

212 Next, we quantified viral RNA and infectious virus in all control and thermal treated
213 samples. The qRT-PCR results revealed comparable levels of viral RNA in both heat-treated and
214 non-treated milk samples (**Fig. 4d**). Virus quantifications in Cal-1 cells or ECEs demonstrated viral
215 titers of 4.66 ± 0.19 log $TCID_{50}$/mL and 4.54 ± 0.03 log EID_{50}/mL in untreated milk samples,
216 which decreased by one log in milk samples heat treated at 50°C for 10 min, with titers of 3.69 ±
217 0.38 log $TCID_{50}$/mL and 3.5 log EID_{50}/mL (**Fig. 4e-f**). Notably, no infectious virus titers were
218 detected in milk samples heat treated at 60°C for 10 min, 63°C for 22 s, 63°C for 30 min, 69°C for
219 22 s and 72°C for 15 s. These results corroborate the findings of our studies using a thermocycler
220 and demonstrate the efficacy of a broad spectrum of thermal treatment conditions on inactivation
221 of HPAI H5N1 virus in raw milk.

222 **Kinetics of HPAI H5N1 virus inactivation by thermal treatment in raw milk.** We evaluated
223 the kinetics of thermal inactivation of HPAI H5N1 virus in raw milk at different temperatures over
224 time using the submerged coil system. For this, raw HPAI-spiked milk was subjected to the thermal
225 treatment and samples were collected at different time points as follows: 50°C - 5, 10, 15 20, 25,
226 and 30 min; 60°C - 5, 10, 25, 20, 30, and 60 sec; 63°C - 5, 10, 15, 20, and 30 sec; and 72°C - 5, 7,
227 10, 12, and 15 sec. Viral RNA and infectious virus were quantified by qRT-PCR and virus titrations
228 (cells and ECEs), respectively. Viral RNA levels detected in untreated and heat-treated milk
229 samples were comparable in all temperatures evaluated in the study (**Fig. 5a, d, g, j**). At 50°C, we
230 observed a gradual decrease in infectious virus titers from 4.88 ± 0.14 to 2.30 ± 0.25 log
231 $TCID_{50}$/mL within 25 min heat treatment with virus titers falling under the titration detection limit

232 between the 25- and 30-min time point (**Fig. 5b**). Similar results were observed in ECEs with viral
233 virus titers decreasing from 4.58 ± 0.07 to 1.17 ± 1.01 log EID_{50}/mL within 25 min (**Fig. 5c**). Of
234 note, one out of three milk samples heat treated at 50°C for 30 min showed embryo mortality and
235 HA activity, indicating the presence of low amounts of infectious virus in this sample. The D-value
236 of HPAI H5N1 at 50°C was estimated at 10.11 min. Notably, rapid virus inactivation of HPAI
237 H5N1 was observed in raw milk samples heat treated at 60°C (**Fig. 5e, 5f**), 63°C (Fig. **5h, 5i**) and
238 72°C (**Fig. 5k, 5l**), with heating for 5 sec leading to complete virus inactivation at these
239 temperatures. These results demonstrate that HPAI H5N1 virus is stable for at least 30 min at 50°C
240 in raw milk. Importantly, however, efficient and rapid inactivation (within five seconds) of HPAI
241 H5N1 virus is achieved in raw milk heat treated at temperatures of 60°C or above.

242 **Discussion**

243 The high tropism of HPAI H5N1 virus and its replication in milk secreting epithelial cells of the
244 mammary gland in dairy cows, lead to high viral loads and shedding (from $10^{4.4}$ to up $10^{8.8}$
245 $TCID_{50}.mL^{-1}$) in milk[6,7,15]. Despite the widespread use of milk pasteurization by the dairy industry,
246 the high viral loads detected in milk from infected cows raised major public health concerns.
247 Although it has been shown that both high (HPAI)- and low pathogenic avian influenza (LPAI)
248 viruses can be inactivated by pasteurization of various poultry products including fat-free egg
249 products, allantoic fluid and plasma[16], and milk coproduct concentrated lactose[17], in the beginning
250 of the dairy outbreak there was no data demonstrating the efficacy of pasteurization on HPAI H5N1
251 virus in dairy products. Initial laboratory studies evaluating inactivation of HPAI in raw milk
252 revealed that the most used FDA approved milk pasteurization condition (72°C for 15 sec)
253 markedly reduced viral loads in raw milk, however, low amounts of infectious virus were still
254 detected in heat treated samples[12,18]. Here we evaluated the stability and determined the decay of
255 HPAI H5N1 virus in raw milk stored at different temperatures over time and investigated the
256 efficacy and kinetics of inactivation of HPAI H5N1 virus following the two FDA approved
257 pasteurization- as well as various thermization (subpasteurization) conditions widely used by the
258 dairy industry.

259 Results from our decay studies revealed long-term stability of HPAI H5N1 in raw clinical
260 milk samples collected from HPAI H5N1 infected cows and stored at 4°C (refrigeration
261 temperature), with infectious virus being detected for up to 56 days (8 weeks) in these samples.

262 These results corroborate findings of a recent study in which infectious HPAI H5N1 was recovered
263 for up to 5 weeks from milk samples spiked with the virus[12], highlighting the potential public
264 health risk posed by consumption of raw milk and other raw milk derived products such as raw
265 milk cheeses which must be aged for 60 days at 4°C[19] prior to human consumption. Survival of
266 HPAI H5N1 virus in raw milk incubated at 4°C for 56 days with residual titers of about 3.5-3.8 log
267 $EID_{50}.ml^{-1}$ as shown here (**Extended Data Fig. 1a**), underscore the need for further studies to
268 determine whether the aging process efficiently inactivates HPAI H5N1 virus in raw milk cheeses.
269 The decay studies performed here provide important information regarding the sensitivity of HPAI
270 H5N1 to higher temperatures. Incubation of raw clinical milk from infected cows at 20°C resulted
271 in inactivation of the virus within 21 days (3 weeks), whereas rapid virus decay was observed at
272 30°C (6 days, <1 week) and 37°C (2 days) in spiked milk samples. Therefore, thermal treatment
273 of milk from infected cows to temperatures between 30-37°C, could potentially be utilized to
274 inactivate HPAI H5N1 virus in raw milk prior to its disposal in affected farms. This would
275 minimize the risk of environmental contamination and further virus spread.

276 Although the grade A pasteurized milk ordinance (PMO)[20] requires that milk from sick
277 animals is segregated on the farm, FDA and USDA found PCR positive shelf milk samples[11],
278 indicating that the virus found its way into bulk raw milk making it into grocery stores. Importantly,
279 follow up testing of these samples to determine virus infectivity have shown that no infectious
280 virus was present in these PCR positive samples[11], which is likely a result of pasteurization. While
281 early laboratory studies have shown that heat treatment of HPAI H5N1 spiked milk samples to
282 conditions that mimic FDA approved pasteurization conditions (63°C for 30 min and 72°C for 15
283 sec) results in drastic reduction in viral titers, residual virus infectivity was still detected when
284 these samples were subjected to the thermal treatment at 72°C for 15 sec[12,18]. Results here,
285 however, using raw milk from HPAI infected cows or raw milk spiked with HPAI H5N1 virus,
286 show that the two FDA approved pasteurization conditions efficiently inactivated the virus
287 resulting in up to 7.3 log EID_{50} reduction in virus infectivity. These findings corroborate a study
288 conducted by USDA and FDA which showed that HTST in a lab-scale continuous flow pasteurizer
289 efficiently inactivated HPAI H5N1 in raw milk[21]. Together, these findings demonstrate that
290 pasteurization is highly effective in reducing the load of HPAI H5N1 virus in milk.

291 We further extended our thermal inactivation studies to include four thermization
292 conditions that are frequently used in dairy industry for production of some cheeses[13,14]. Three out

293 of four thermization conditions tested including 60°C for 10 min, 63°C for 22 s and 69°C for 22 s
294 resulted in inactivation of HPAI in raw milk. Thermization of raw milk at 50°C for 10 min,
295 however, only partially reduced HPAI H5N1 virus titers (0.83-2.42 EID_{50} log reduction). Together
296 these results demonstrate that HPAI H5N1 is highly sensitive to inactivation in raw milk subjected
297 to thermal treatment at temperatures on and above 60°C. This was confirmed by our inactivation
298 kinetics studies. Based on our calculated D-value at 50°C, it would take 50.55 minutes to achieve
299 a 5-log reduction in HPAI H5N1 loads in contaminated raw milk. In FDA's survey of bulk milk
300 tanks, they observed and average 3.5 log EID_{50} load, with a range of 1.3 to 6.3 log EID_{50}[21]. A 10°C
301 increase in the treatment temperature to 60°C resulted in rapid virus inactivation within 5 seconds
302 of treatment, and while the inactivation was too fast to capture shorter-time intervals, the
303 approximated D-value is 1.04s (1/ (4.8 log EID_{50}/5)), assuming a linear decay over 5 seconds,
304 highlighting the efficacy of thermal treatment in ensuring safety of milk and other dairy products
305 derived from pasteurized milk. Additionally, several of the thermization conditions tested in our
306 controlled experimental settings proved effective against HPAI H5N1, suggesting the use of
307 thermized milk treated at 60°C and above for 15-20 seconds to produce cheeses would reduce the
308 risk of human exposure to products with infectious HPAI H5N1.

309 In summary, our study provides a comprehensive overview of the thermal inactivation
310 spectrum of HPAI H5N1 virus in raw milk, demonstrating the efficacy of thermal treatment
311 including thermization and pasteurization conditions on inactivation of HPAI H5N1 virus in raw
312 milk.

313 **Materials and Methods**

314 **Cells and virus**

315 Bovine uterine epithelial cells (Cal-1, developed in house at the Virology Laboratory at AHDC)
316 were cultured in minimal essential medium (MEM, Corning Inc., Corning, NY) supplemented with
317 10% fetal bovine serum (FBS) and penicillin-streptomycin (ThermoFisher Scientific, Waltham,
318 MA; 10 $U.mL^{-1}$ and 100 $\mu g.mL^{-1}$, respectively). The HPAI H5N1 TX2/24
319 (A/Cattle/Texas/063224-24-1/2024, genotype B3.13, GISAID accession number:
320 EPI_ISL_19155861) isolated from the milk of infected dairy cattle in Texas, USA[6] was used in
321 the decay and pasteurization studies. The virus stock was propagated and titrated in Cal-1 cells.

322 **Biosafety and biosecurity**

323 All work involving handling and propagation of HPAI was performed following strict biosafety
324 measures in AHDC BSL3 laboratories of College of Veterinary Medicine, Cornell University.

325 **Milk samples**

326 Normal raw milk samples were obtained from Teaching Dairy at Cornell University and used as
327 negative control as well as the matrix to be spiked with HPAI H5N1 virus during heat treatment
328 studies. Raw clinical milk samples used in the decay and thermal treatment studies were collected
329 by field veterinarians from a clinically affected dairy farm in Texas, United States [6]. A total of 15
330 milk samples were pooled and used in decay study (Fig. 1a-b) and heat treatment studies (Fig. 2).

331 **Thermal decay of HPAI H5N1 virus in milk**

332 For thermal decay studies, raw clinical milk samples from HPAI infected dairy cows and/or HPAI-
333 spiked raw milk samples were used. For raw clinical milk samples, 15 IAV M RT-PCR positive
334 milk samples from HPAI infected dairy cows from Texas were pooled and 45 vials of 1 ml aliquots
335 were prepared. The raw clinical milk samples were incubated at 4°C and 20°C. For HPAI-spiked
336 samples, raw normal milk samples obtained from Cornell Teaching Dairy were spiked with HPAI
337 H5N1 TX2/24 (stock titer $2x10^7$ log $TCID_{50}$/mL) at 1:20 dilution to achieve a final titer of 6 log
338 $TCID_{50}$/mL and 45 vials of 1 ml aliquots were prepared. The HPAI-spiked milk samples were then
339 incubated at 30°C and 37°C. For all temperatures, sequential samples (n=3 per sampling point)
340 were collected on days 0, 1, 2, 3, 4, 5, 6, 7, 14, 21, 28, 35, 42, 49 and 56 and stored at -80°C for
341 virus quantifications.

342 **Thermal inactivation of HPAI in raw milk using a thermocycler**

343 Initially, the thermal treatment of raw milk to assess inactivation of HPAI H5N1 was performed
344 using a thermocycler. Both pooled raw clinical milk samples from HPAI infected cows and HPAI-
345 spiked raw milk samples were used. For HPAI-spiked samples, raw normal milk obtained from the
346 Cornell Teaching Dairy were spiked with HPAI H5N1 TX2/24 (stock titer $2x10^7$ log $TCID_{50}$/mL)
347 at 1:20 ratio to achieve a target titer of 6 log $TCID_{50}$/mL. For heat treatment, we used combinations
348 of six different temperatures and holding times that are used for thermal processing of milk and
349 milk products in dairy industry. We tested two pasteurization conditions, low temperature long

350 time (LTLT) at 63°C 30 min and high temperature short time (HTST) at 72°C 15 s as well as four
351 thermization conditions of 50°C for 10 min, 60°C for 10 min, 63°C for 22 sec and 69°C for 22 sec.
352 For heat treatment, 100 µL/tube of raw clinical milk or HPAI-spiked raw normal milk samples
353 were added in 8-tube PCR strip. A total of 4 strips (3.2 mL milk) were used per replicate. Following
354 heat treatment in a thermocycler (BioRad T100 Thermal Cycler), milk samples were chilled
355 immediately on ice for 10 min. The milk samples were pooled, three aliquots of 1 ml volume were
356 prepared for each replicate and stored at -80°C until further testing.

357 **Heat treatment of milk using a submerged coil heating system**

358 To more closely simulate pasteurization conditions, we developed a submerged coil heating
359 system. The stainless-steel coil tubing (FITOK 316/316L Stainless Steel Seamless Tubing, 1/8"
360 OD × 0.028" Wall, Coil) system has four segments (Fig. 4a) that are furnished with four
361 temperature sensors (Quick Disconnect Thermocouples with Miniature Connectors, Omega
362 Engineering Inc., USA) at each segment. The first segment consists of two injection ports for
363 sample and water and is partly submerged into a water bath. The second and third loops are
364 completely submerged in water and hold the desired temperature immediately after the sample
365 injection. The fourth segment is the exit loop for sample collection and is partly submerged into
366 the water. The thermal sensors are connected to a data logger (HH378, Data Logger/K, J, E, T,
367 Type Thermometer, Omega Engineering Inc, USA) and temperatures are recorded throughout the
368 heat treatment process. A circulating water bath (Cole-Parmer EW-16101-89: Unheated Water
369 Bath, Fisher Scientific) furnished with Immersion Circulators (VWR) was used to heat the coils.
370 About 15 mL of milk samples were injected into the sample port by using a 20 mL syringe. After
371 incubation at the desired temperature and time, milk samples (1-5 mL) were pushed out of the coil
372 system by injecting sterile water in the system. Each milk sample was collected into a sterile tube.
373 Immediately after collection, the milk samples were chilled on ice for 10 min, aliquoted and stored
374 at -80°C. After each round, the coil was cleaned by flushing the coil with a 1.5% w/v solution of
375 alkaline detergent (Alcanox, Sigma-Aldrich) followed by 1% w/v solution of acid detergent
376 (Citranox, Alconox) and finally passing several coil-full volumes of sterile water and by passing
377 the air several times.

378 **RNA extraction and RT-PCR**

379 Viral RNA from milk samples was extracted using the IndiMag Pathogen kit (INDICAL
380 Bioscience) on the IndiMag 48s automated nucleic acid extractor (INDICAL Bioscience, Leipzig,
381 Germany), and the rRT-PCR was performed using the Path-ID™ Multiplex One-Step RT-PCR Kit
382 (Thermo Fisher, Waltham, MA, USA) and primers and probes targeting the M gene under the
383 following conditions: 15 min at 48ºC, 10 min at 95 °C, 40 cycles of 15 s at 95 °C for denaturation
384 and 60 s at 60 °C. A standard curve was prepared using RNA extracted from HPAI H5N1 TX2/24
385 spiked milk samples. Serial 10-fold dilutions of the stock virus ($2x10^7$ log $TCID_{50}$/mL) were
386 prepared in raw normal milk samples for RNA extraction followed by RT-PCR as described above.
387 The Ct values were used to estimate the viral RNA copy number in the tested milk samples using
388 relative quantification method.

389 **Virus titration in cell culture ($TCID_{50}$)**

390 The infectious viral loads in milk samples were quantified by viral titrations in Cal-1 cells. For
391 this, serial 10-fold dilutions of milk samples were prepared in MEM and inoculated into Cal-1
392 cells in 96-well plates. Each dilution was inoculated in quadruplicate wells. At 48h post-
393 inoculation, culture supernatant was aspirated, and cells were fixed with 3.7% formaldehyde
394 solution for 30 min at RT and subjected to IFA using the anti-NP (HB65) mouse monoclonal
395 antibody, followed by secondary anti-mouse-Alexa-594 incubation. The 50% tissue culture
396 infectious dose ($TCID_{50}$) was determined using end-point dilutions and the Spearman and Karber's
397 method and expressed as $TCID_{50}.mL^{-1}$.

398 **Virus titration in embryonated chicken eggs (EID_{50})**

399 For virus titration in eggs, 9-days-old embryonated chicken eggs were used. Serial 10-fold
400 dilutions of milk samples were prepared in phosphate buffered saline (PBS) supplemented with
401 antibiotic-antimycotic (anti-anti 100X, Thermo Fisher Scientific, USA) at 2:1 ratio. The eggs were
402 candled to mark the air sac on the shell, sanitized with 70% ethanol and a hole was drilled in the
403 eggshell. One hundred µL of the diluted milk sample was injected in quadruplicate into the chorio-
404 allantoic sac route and sealed with glue. Eggs were candled daily for four days, and dead embryos
405 were chilled overnight before collection. After 4 days post-inoculation, all surviving embryos were
406 chilled for 24 hours, and allantoic fluids were collected. All allantoic fluids were tested by
407 hemagglutination assay (HA) using 0.5% chicken RBC. Finally, the 50% egg infectious dose
408 (EID_{50}) was calculated by the Reed and Muench method.

409 **Hemagglutination assay (HA)**

410 For the HA, 100 µL allantoic fluid was taken into the wells of 1st row of a 96-well U-bottom plate.
411 50 µL of PBS was added to the wells of 2nd, 3rd and 4th rows. Three 2-fold dilutions (1:2, 1:4 and
412 1:8) of the allantoic fluids were prepared in PBS. Then, 50 µL of 0.5% chicken red blood cells
413 (RBC) were added to each well and incubated at room temperature for 30 min. Lack of RBC button
414 formation indicates positive HA reaction.

415 **Statistical analysis**

416 Graphs were prepared using GraphPad Prism 10 software. Linear regression analysis was
417 performed to obtain the thermal death time D-values.

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421 help in setting up the protocols and procedures to conduct work with HPAI H5N1 virus at the
422 Cornell BSL-3 facilities and the Cornell Teaching Dairy for proving the raw milk samples. The
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424 coil system for use in this study.

425 **Author contributions**

426 Conceptualization: DGD; Methodology: MN, LMC, KK, RI, NHM, SDA, DGD; Software: MN,
427 KK; Validation: MN, LMC, KK; Formal analysis: MN, LMC, KK, RI, DGD; Investigation: MN,
428 LMC; Resources: NHM, SDA, DGD; Data Curation: MN, LMC, KK; Writing - Original Draft:
429 MN, DGD; Writing - Review & Editing: MN, LMC, KK, NHM, RI, SDA, DGD; Visualization:
430 MN; Supervision: DGD; Project administration: DGD, Funding acquisition: NHM, SDA, DGD.

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434 **References**

435 1. USDA. 2022-2023 Confirmations of Highly Pathogenic Avian Influenza in Commercial and
436 Backyard Flocks. *HPAI 2022/2023 Confirmed Detections* Preprint at
437 https://www.aphis.usda.gov/aphis/ourfocus/animalhealth/animal-disease-
438 information/avian/avian-influenza/hpai-2022/2022-hpai-commercial-backyard-flocks
439 (2023).
440 2. APHIS-USDA. *Detections of Highly Pathogenic Avian Influenza in Mammals*. (2024).
441 3. Elsmo, E. J. *et al.* Highly Pathogenic Avian Influenza A(H5N1) Virus Clade 2.3.4.4b
442 Infections in Wild Terrestrial Mammals, United States, 2022. *Emerg Infect Dis* **29**, (2023).
443 4. AVMA. *Goat in Minnesota Tests Positive for HPAI. Available at:*
444 *Https://Www.Avma.Org/News/Goat-Minnesota-Tests-Positive-Hpai.* (2024).
445 5. Burrough, E. R. *et al.* Highly Pathogenic Avian Influenza A(H5N1) Clade 2.3.4.4b Virus
446 Infection in Domestic Dairy Cattle and Cats, United States, 2024. *Emerg Infect Dis* **30**,
447 (2024).
448 6. Caserta, L. C. *et al.* Spillover of highly pathogenic avian influenza H5N1 virus to dairy
449 cattle. *Nature* (2024) doi:10.1038/s41586-024-07849-4.
450 7. Halwe, N. J. *et al.* Outcome of H5N1 clade 2.3.4.4b virus infection in calves and lactating
451 cows. *bioRxiv* 2024.08.09.607272 (2024) doi:10.1101/2024.08.09.607272.
452 8. Centers for Disease Control and Prevention. H5 Bird Flu: Current Situation. (2024).
453 9. Dash, K. K. *et al.* A comprehensive review on heat treatments and related impact on the
454 quality and microbial safety of milk and milk-based products. *Food Chemistry Advances* **1**,
455 100041 (2022).

456 10. FDA. *Updates on Highly Pathogenic Avian Influenza (HPAI). Available at:*
457 *Https://Www.Fda.Gov/Food/Alerts-Advisories-Safety-Information/Updates-Highly-*
458 *Pathogenic-Avian-Influenza-Hpai*. (2024).
459 11. Spackman, E. *et al.* Characterization of highly pathogenic avian influenza virus in retail
460 dairy products in the US. *J Virol* **98**, (2024).
461 12. Guan, L. *et al.* Cow's Milk Containing Avian Influenza A(H5N1) Virus — Heat Inactivation
462 and Infectivity in Mice. *New England Journal of Medicine* **391**, 87–90 (2024).
463 13. Deeth, H. C. Heat Treatment of Milk: Pasteurization (HTST) and thermization (LTLT). in
464 *Encyclopedia of Dairy Sciences* 645–654 (Elsevier, 2022). doi:10.1016/B978-0-12-818766-
465 1.00133-1.
466 14. Deeth, H. Optimum Thermal Processing for Extended Shelf-Life (ESL) Milk. *Foods* **6**, 102
467 (2017).
468 15. Nelli, R. K. *et al.* Sialic Acid Receptor Specificity in Mammary Gland of Dairy Cattle
469 Infected with Highly Pathogenic Avian Influenza A(H5N1) Virus. *Emerg Infect Dis* **30**,
470 (2024).
471 16. Pitino, M. A., O'Connor, D. L., McGeer, A. J. & Unger, S. The impact of thermal
472 pasteurization on viral load and detectable live viruses in human milk and other matrices: a
473 rapid review. *Applied Physiology, Nutrition, and Metabolism* **46**, 10–26 (2021).
474 17. Kwon, T. *et al.* Bovine Highly Pathogenic Avian Influenza Virus Stability and Inactivation
475 in the Milk Byproduct Lactose. *Viruses* **16**, 1451 (2024).
476 18. Kaiser, F. *et al.* Inactivation of Avian Influenza A(H5N1) Virus in Raw Milk at 63°C and
477 72°C. *New England Journal of Medicine* **391**, 90–92 (2024).
478 19. 21 CFR Part 133. Cheeses and Related Cheese Products. *CFR* (2024).

20. USPHS/FDA. *Grade A Pasteurized Milk Ordinance (PMO)*. (2023).

21. Spackman, E. *et al.* Inactivation of highly pathogenic avian influenza virus with high temperature short time continuous flow pasteurization and virus detection in bulk milk tanks. *medRxiv* (2024) doi:10.1101/2024.07.01.24309766.

Extended Data Table 1. Isolation of HPAI H5N1 virus in raw clinical milk collected from HPAI infected cows and HPAI-spiked raw normal milk incubated at different temperatures by using embryonated chicken eggs.

	Cal-1	Chicken embryos				Cal-1	Chicken embryos		
4°C D49		Dilutions	Death	HA (+)	**4°C D56**		Dilutions	Death	HA (+)
R1	Toxicity	1:10	2/3	2/3	R1	Toxicity	1:10	3/3	3/3
		$1:10^2$	1/3	1/3			$1:10^2$	3/3	3/3
		$1:10^3$	0/3	0/3			$1:10^3$	0/3	0/3
R2	Toxicity	1:10	2/3	2/3	R2	Toxicity	1:10	3/3	3/3
		$1:10^2$	1/3	1/3			$1:10^2$	3/3	3/3
		$1:10^3$	0/3	0/3			$1:10^3$	0/3	0/3
R3	Toxicity	1:10	3/3	3/3	R3	Toxicity	1:10	3/3	3/3
		$1:10^2$	3/3	3/3			$1:10^2$	3/3	3/3
		$1:10^3$	0/3	0/3			$1:10^3$	0/3	0/3
20°C D14					**20°C D21**				
R1	Toxicity	1:10	1/3	1/3	R1	Toxicity	1:10	1/3	0/3
		$1:10^2$	0/3	0/3			$1:10^2$	0/3	0/3
		$1:10^3$	0/3	0/3			$1:10^3$	0/3	0/3
R2	Toxicity	1:10	1/3	0/3	R2	Toxicity	1:10	2/3	0/3
		$1:10^2$	0/3	0/3			$1:10^2$	0/3	0/3
		$1:10^3$	0/3	0/3			$1:10^3$	0/3	0/3
R3	Toxicity	1:10	1/3	0/3	R3	Toxicity	1:10	1/3	0/3
		$1:10^2$	0/3	0/3			$1:10^2$	0/3	0/3
		$1:10^3$	0/3	0/3			$1:10^3$	0/3	0/3
30°C D6					**30°C D7**				
R1	Toxicity	1:10	0/3	0/3	R1	Toxicity	1:10	0/3	0/3
		$1:10^2$	0/3	0/3			$1:10^2$	0/3	0/3
		$1:10^3$	0/3	0/3			$1:10^3$	0/3	0/3
R2	Toxicity	1:10	0/3	0/3	R2	Toxicity	1:10	0/3	0/3
		$1:10^2$	0/3	0/3			$1:10^2$	0/3	0/3
		$1:10^3$	0/3	0/3			$1:10^3$	0/3	0/3
R3	Toxicity	1:10	0/3	0/3	R3	Toxicity	1:10	0/3	0/3
		$1:10^2$	0/3	0/3			$1:10^2$	0/3	0/3
		$1:10^3$	0/3	0/3			$1:10^3$	0/3	0/3
37°C D2					**37°C D3**				
R1	Toxicity	1:10	0/3	0/3	R1	Toxicity	1:10	0/3	0/3
		$1:10^2$	0/3	0/3			$1:10^2$	0/3	0/3
		$1:10^3$	0/3	0/3			$1:10^3$	0/3	0/3
R2	Toxicity	1:10	0/3	0/3	R2	Toxicity	1:10	0/3	0/3
		$1:10^2$	0/3	0/3			$1:10^2$	0/3	0/3
		$1:10^3$	0/3	0/3			$1:10^3$	0/3	0/3
R3	Toxicity	1:10	0/3	0/3	R3	Toxicity	1:10	0/3	0/3
		$1:10^2$	0/3	0/3			$1:10^2$	0/3	0/3
		$1:10^3$	0/3	0/3			$1:10^3$	0/3	0/3

Note: D, days; R, replicates; HA, hemagglutination assay; +, positive

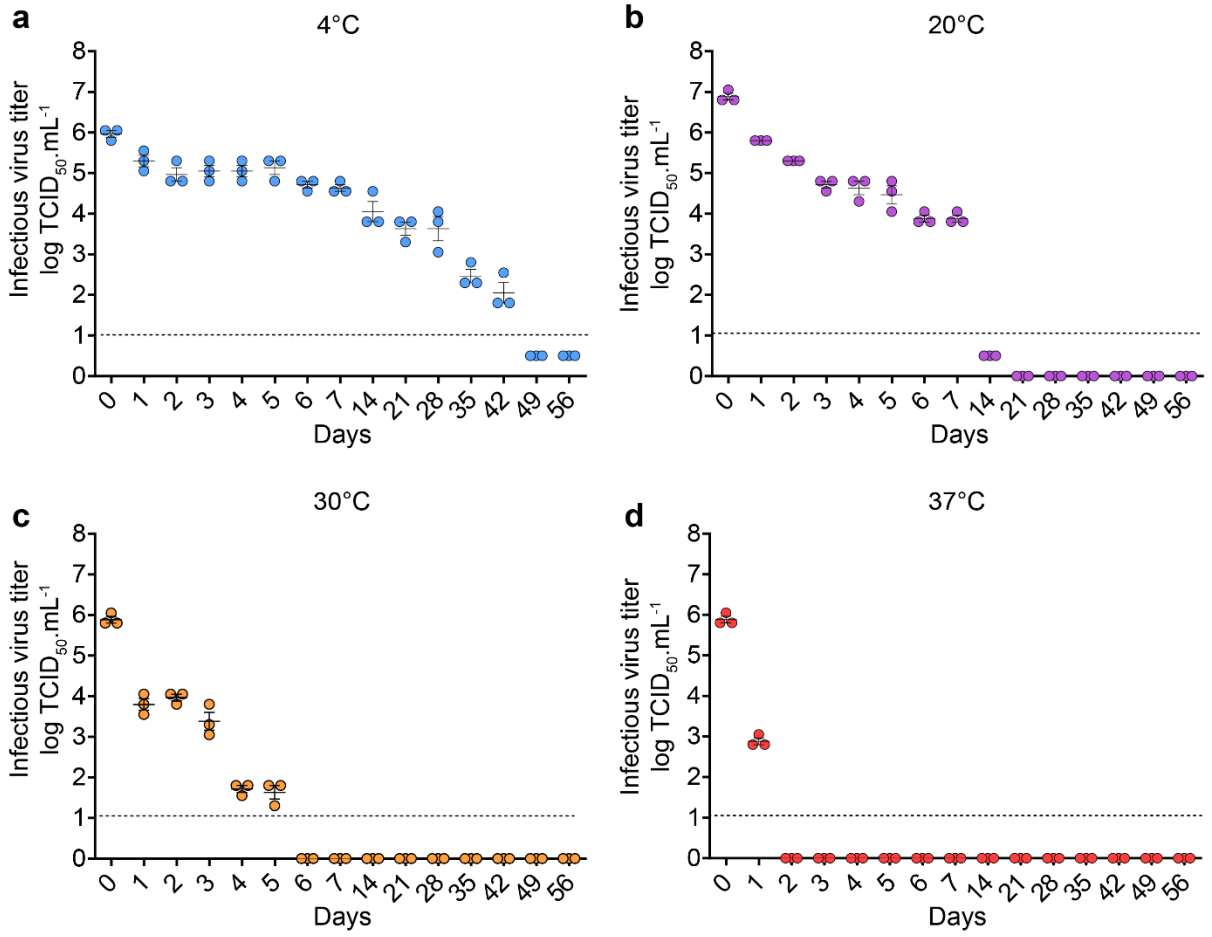


Fig. 1. Kinetics HPAI H5N1 virus decay in milk. Decay of HPAI H5N1 virus in raw clinical milk collected from HPAI infected cows incubated at 4°C (**a**) and 20°C (**b**) for 56 days. Decay of HPAI H5N1 virus in HPAI-spiked (1:20 ratio) raw normal milk and incubated at 30°C (**c**) and 37°C (**d**) for 56 days. The virus titers were determined by end point titrations in Cal-1 cells (Log $TCID_{50}.mL^{-1}$). Data represents observations (dots) from three replicates (n=3) overlayed with the mean (horizontal line) and ± SEM (whiskers).

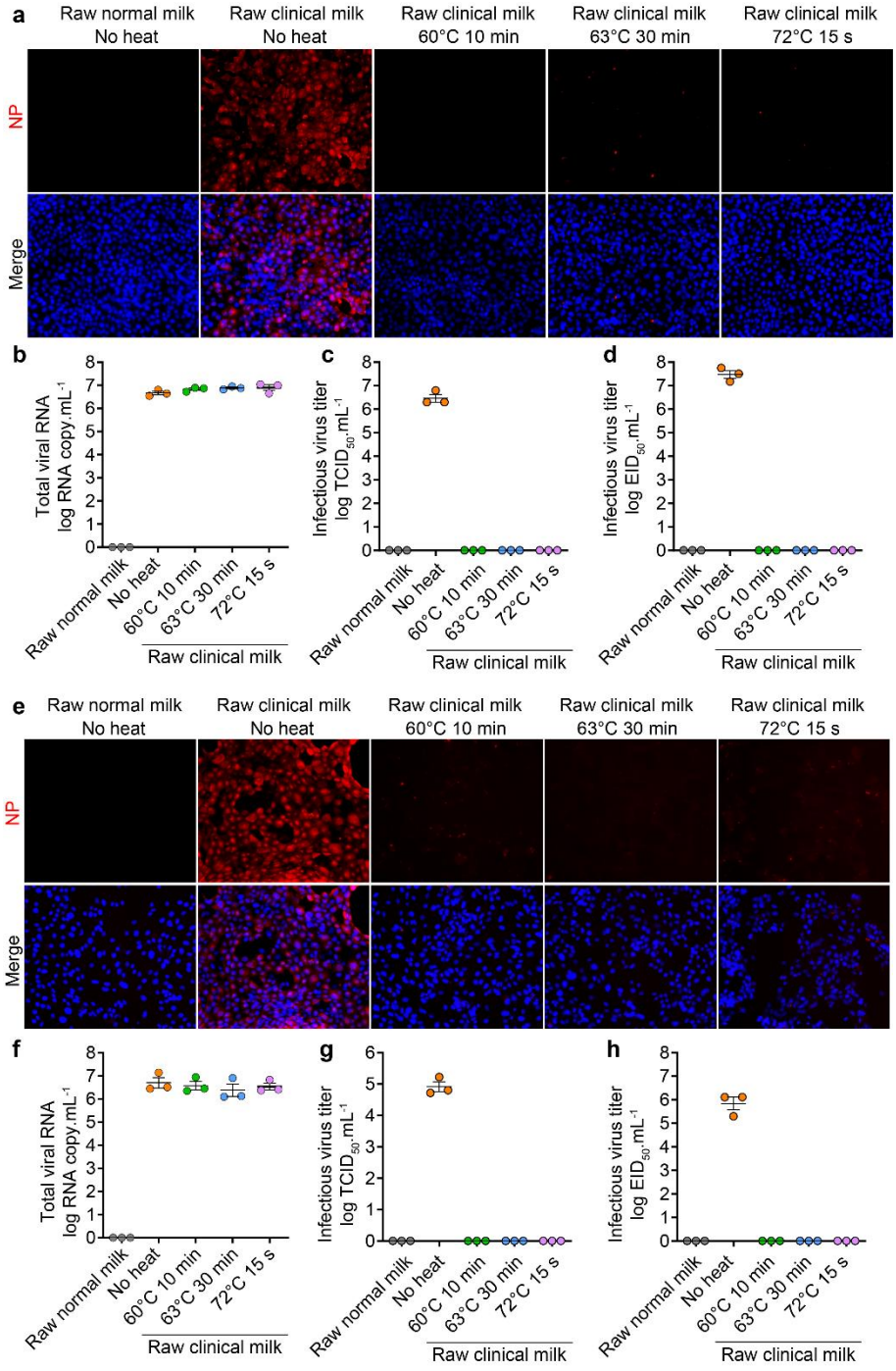


Fig. 2. Effect of low temperature thermization and FDA approved pasteurization conditions on HPAI H5N1 infectivity in raw milk. **a-d.** Thermal inactivation of HPAI H5N1 virus in raw clinical milk samples collected from HPAI infected cows. **a.** Immunofluorescence (IFA) images of Cal-1 cells inoculated with control (normal and clinical) or heat-treated clinical milk samples. Raw clinical milk samples were collected from HPAI H5N1 infected cows and heat treated at the indicated temperatures and times utilizing a thermocycler. **b.** HPAI H5N1 viral RNA loads in control or heat-treated raw clinical milk samples as determined by qRT-PCR (n = 3). **c.** HPAI H5N1 virus titrations in Cal-1 cells (n = 3). **d.** HPAI H5N1 virus titrations in embryonated chicken eggs

(n = 3). **e-h.** Thermal inactivation of HPAI H5N1 virus in raw normal milk spiked (1:10 ratio) with milk samples collected from HPAI infected cows using a thermocycler. **e.** IFA images of Cal-1 cells inoculated with control (normal and clinical) or heat-treated spiked milk samples. **f.** HPAI H5N1 viral RNA loads in control or heat-treated raw spiked milk samples as determined by qRT-PCR (n = 3). **g.** HPAI H5N1 virus titrations in Cal-1 cells (n = 3). **h.** HPAI H5N1 virus titrations in embryonated chicken eggs (n = 3). Red fluorescence indicates detection of the viral NP protein. Images are representative of three independent experiments (n = 3). The virus titers were determined by end point dilutions in Cal-1 cells and in embryonated chicken eggs and expressed as tissue culture infectious dose 50 per mL ($TCID_{50}.mL^{-1}$) and egg infectious dose 50 per ml ($EID_{50}.mL^{-1}$), respectively. Data represents observations (dots) from three independent experiments (n=3) overlayed with the mean (horizontal line) and ± SEM (whiskers).

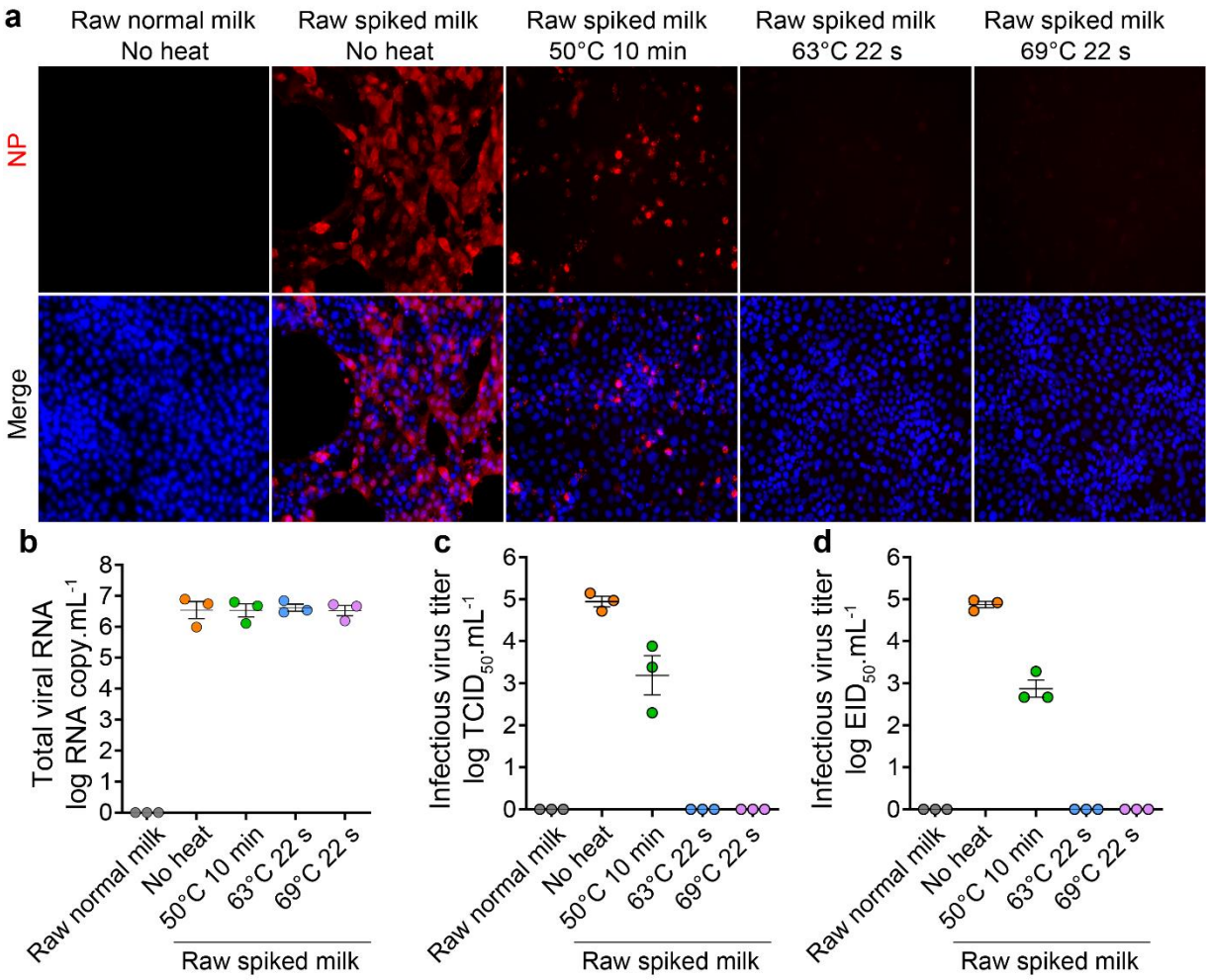


Fig. 3. Evaluation of thermization conditions on inactivation of HPAI H5N1 virus in raw milk. Thermal inactivation of HPAI H5N1 virus in raw normal milk spiked (1:20 ratio) with HPAI H5N1 using a thermocycler. **a.** IFA images of Cal-1 cells inoculated with control or heat-treated HPAI-spiked raw normal milk samples. **b.** HPAI H5N1 viral RNA loads in control or heat-treated HPAI-spiked raw milk samples as determined by qRT-PCR (n = 3). **c.** HPAI H5N1 virus titrations in Cal-1 cells (n = 3). **d.** HPAI H5N1 virus titrations in embryonated chicken eggs (n = 3). Red fluorescence indicates detection of the viral NP protein. Images are representative of three independent experiments (n = 3). The virus titers were determined by end point dilutions in Cal-1 cells and in embryonated chicken eggs and expressed as tissue culture infectious dose 50 per mL ($TCID_{50}.mL^{-1}$) and egg infectious dose 50 per ml ($EID_{50}.mL^{-1}$), respectively. Data represents observations (dots) from three independent experiments (n=3) overlayed with the mean (horizontal line) and ± SEM (whiskers).

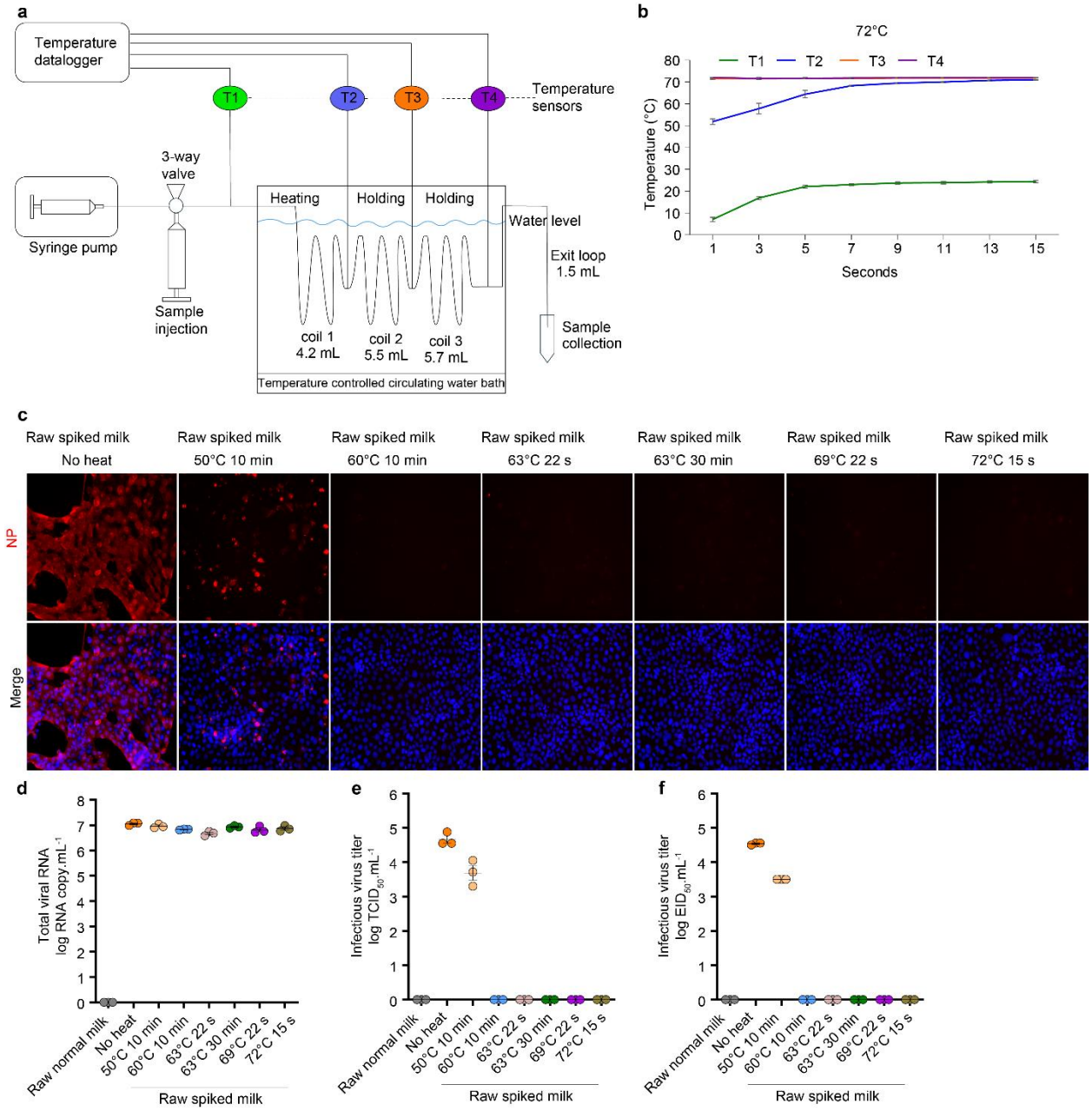


Fig. 4. Thermal inactivation of HPAI H5N1 in virus-spiked milk samples using a submerged coil heating system. **a**. Schematic representation of the submerged coil heating system. **b.** Representative plot showing temperature changes over time in milk samples injected in the submerged coil system during heat treatment at 72°C for 15 sec. Data are presented as the mean ± SEM (whiskers) of 3 independent experiments and a line connecting the means. **c-f.** Thermal inactivation of HPAI H5N1 virus in raw normal milk spiked (1:20 ratio) with HPAI H5N1 using a submerged coil heating system. **c.** IFA images of Cal-1 inoculated with control or heat-treated HPAI-spiked raw normal milk samples. **d.** HPAI H5N1 viral RNA loads in control or heat-treated HPAI-spiked raw milk samples as determined by qRT-PCR (n = 3). **e.** HPAI H5N1 virus titrations in Cal-1 cells (n = 3). **f.** HPAI H5N1 virus titrations in embryonated chicken eggs (n = 3). Red fluorescence indicates detection of the viral NP protein. Images are representative of three

55 independent experiments (n = 3). The virus titers were determined by end point dilutions in Cal-1
56 cells and in embryonated chicken eggs and expressed as tissue culture infectious dose 50 per mL
57 ($TCID_{50}.mL^{-1}$) and egg infectious dose 50 per ml ($EID_{50}.mL^{-1}$), respectively. Data represents
58 observations (dots) from three independent experiments (n=3) overlayed with the mean (horizontal
59 line) and ± SEM (whiskers).
60

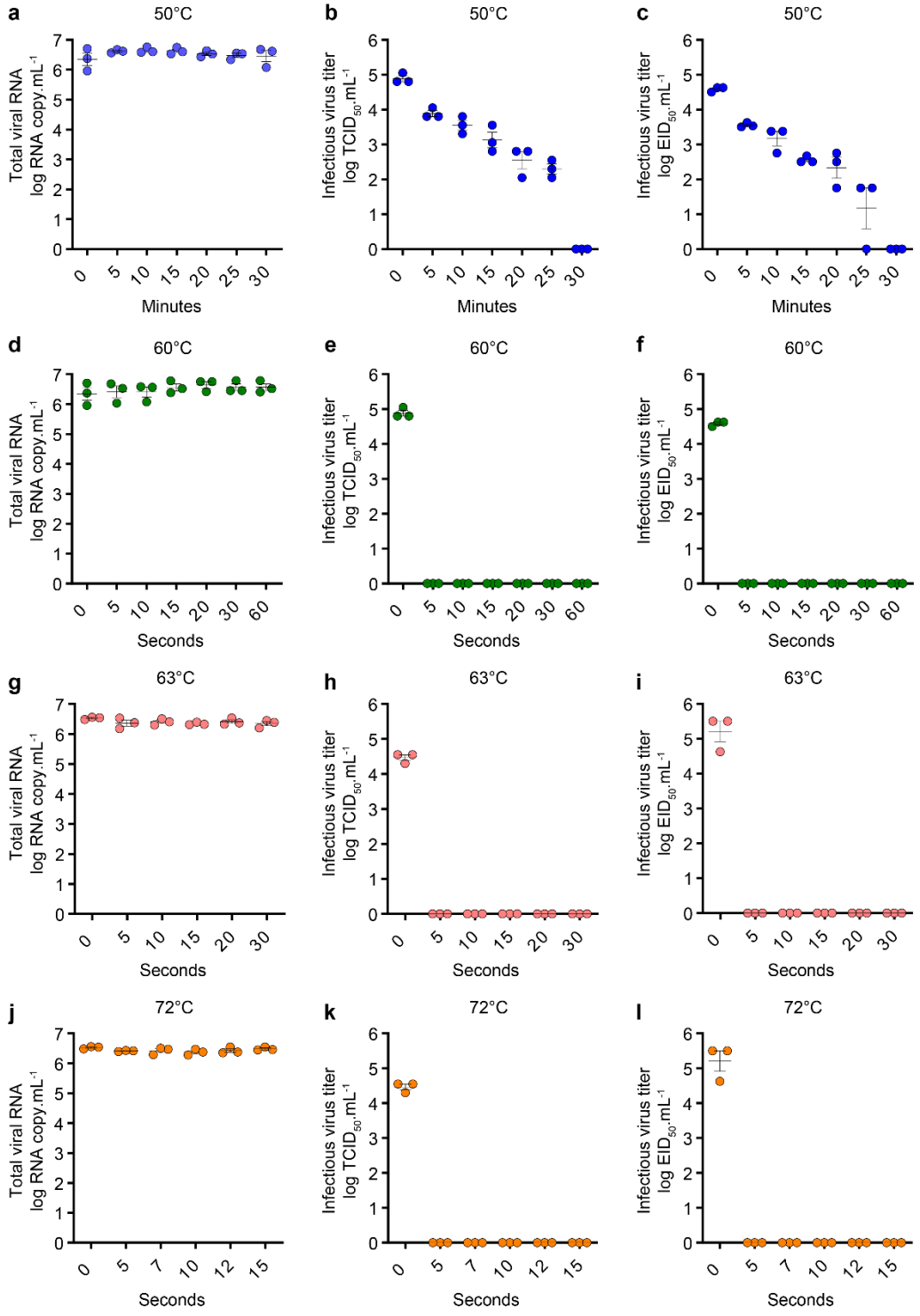
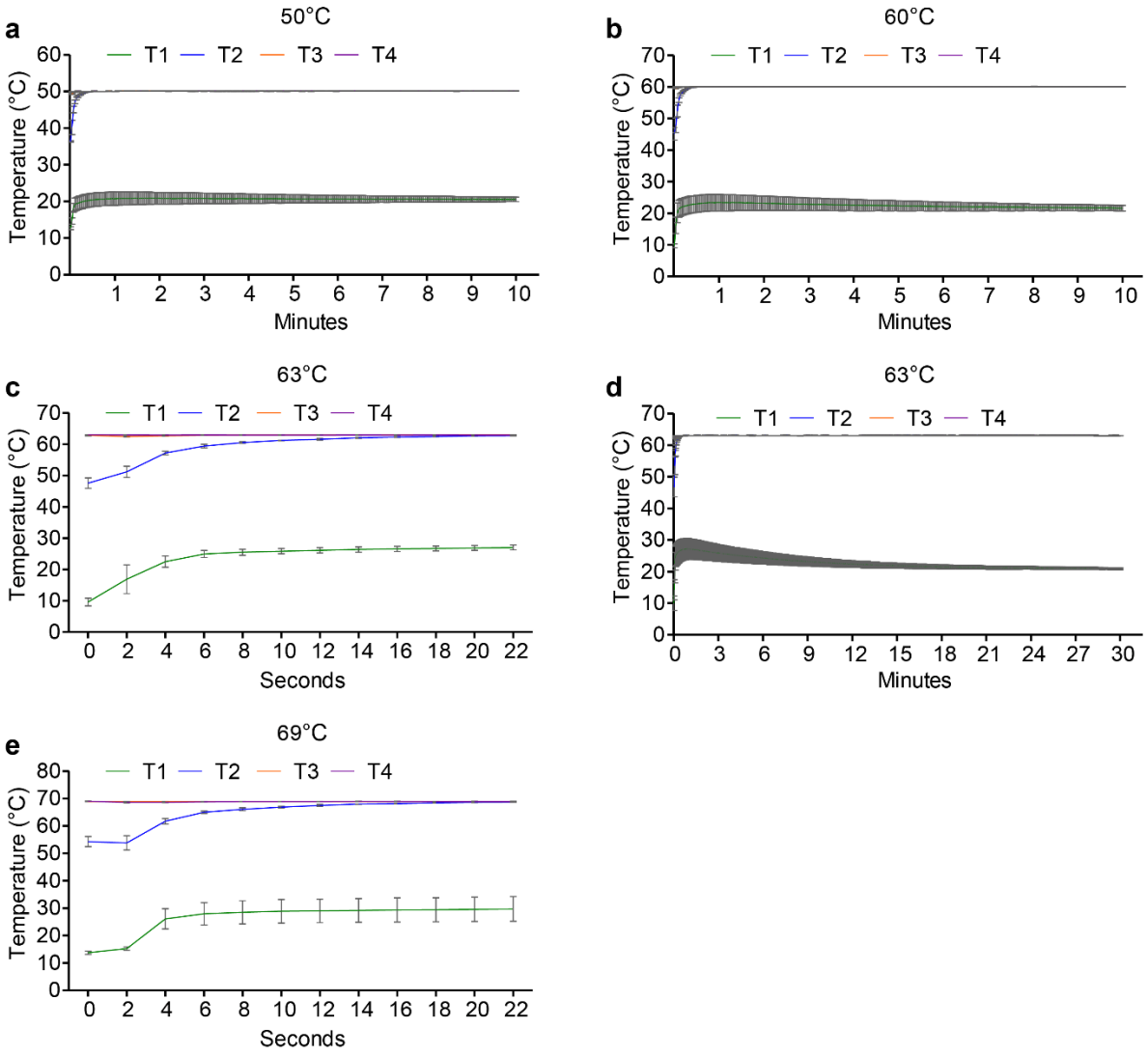


Fig. 5. Kinetics of HPAI H5N1 virus inactivation in milk using submerged coil heating system. Raw normal milk was spiked with HPAI H5N1 at 1:20 ratio and heat treated at 50°C (**a-c**), 60°C (**d-f**), 63°C (**g-i**) and 72°C (**j-l**) with a submerged coil heating system. Sequential samples were collected at the indicated time points and analyzed by qRT-PCR and virus titration in cell culture and embryonated chicken eggs. The virus titers were determined by end point titration in Cal-1

67 cells ($TCID_{50}.mL^{-1}$) and embryonated chicken eggs ($EID_{50}.mL^{-1}$). Data represents observations
68 (dots) from three independent experiments (n=3) overlayed with the mean (horizontal line) and ±
69 SEM (whiskers).
70



Extended Data Fig. 1. Line graph showing temperature changes over time in injected milk samples during heat treatment in the submerged coil heating apparatus at 50°C for 10 min (a), 60°C for 10 min (b), 63°C for 22 s (c), 63°C for 30 min (d) and 69°C for 22 s (e). Data are presented as mean ± SEM of 3 independent experiments.