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 1,314 poultry flocks in 50 states resulting in the death or culling of more than 133 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 29 species confirmed positive to date[2,3], including important livestock species such as goats and dairy cows [4–7]. Spillover of HPAI H5N1 virus to dairy cows and detection of high infectious viral loads and shedding in milk from infected cows[6,8] raised public health concerns given the potential for zoonotic spillover of the virus. As of January18, 2025, there have been 67 confirmed human cases in the USA, with 2024 [9]. 37 of these cases linked to occupational exposures to dairy cows, while 21 were linked to exposures to poultry. Additionally, one human 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[9]. Importantly, the first death due to HAPI H5N1 virus infection was reported in January, 2025 in Louisiana.

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[10]. 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[11,12], coupled with laboratory studies suggesting that heat treatment of raw HPAI H5N1 positive milk at 72ºC for 15 sec, the most common pasteurization method used in the USA, did not completely inactivate the virus[13] 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[12]. 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.

82 Notably, while thermization reduces the number of non-sporeforming pathogens and
83 psychrotrophic spoilage bacteria, it does not destroy all pathogenic microorganisms present in
84 milk[14,15].

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

89 **Results**

90 **Highly pathogenic avian influenza (HPAI) H5N1 virus presents long term stability in raw**
91 **milk at 4ºC but is rapidly inactivated at higher temperatures.** We studied the decay of HPAI
92 H5N1 virus in raw milk at different temperatures. For this, we incubated HPAI H5N1 virus positive
93 raw milk collected from infected cows or normal raw milk spiked with HPAI H5N1 virus at 4°C,
94 20°C, 30°C and 37°C and collected sequential samples daily between days 1-7 and weekly
95 thereafter until day 56 (8 weeks). HPAI H5N1 virus decay over time was assessed by viral titrations
96 performed in bovine uterine epithelial cells (Cal-1, a cell line that his highly susceptible to HPAI
97 H5N1 virus replication when compared to other relevant cells such as primary bovine mammary
98 epithelial cells [bMEC], primary chicken embryo fibroblast [CEF] and Madin-Darby canine
99 kidney [MDCK] cells; **Extended Data Fig. 1**). Samples from select time points that were toxic or
100 negative in the cell culture titration assay were subjected to confirmatory virus isolation in
101 embryonated chicken eggs (ECEs). Virus titers were used to estimate the thermal death time values
102 (D-values) in each studied temperature.

103 Incubation of HPAI H5N1 virus-positive clinical raw clinical milk at 4°C, resulted in a 4-
104 log reduction in infectious virus titers from 5.97 ± 0.14 log $TCID_{50}$/mL on day 0 to 2.05 ± 0.43 log
105 $TCID_{50}$/mL on day 42 **(Fig. 1a)**. Samples from days 49 and 56 were toxic to cell culture, but when
106 inoculated in ECEs both samples were confirmed to contain infectious HPAI H5N1 virus
107 (**Supplementary Table 1**), demonstrating the long-term stability of the virus in raw clinical milk
108 stored 4°C. The *D*-value of HPAI in raw clinical milk stored at 4°C was estimated to be 10.74
109 days. At 20°C, we observed 3-log reduction in infectious virus titers within seven days, with titers
110 dropping from 6.08 ± 0.79 log $TCID_{50}$/mL on day 0 to 2.99 ± 0.77 log $TCID_{50}$/mL on day 7 **(Fig.**
111 **1b)**. Samples collected on days 14 and onward were toxic to cell culture and, thus, were subjected

112 to inoculation in ECEs. All three milk samples collected on day 14 led to embryo mortality, which
113 was confirmed to be caused by HPAI H5N1 virus by hemagglutination (HA) assay
114 (**Supplementary Table 1**). In addition, one out of three samples collected on day 21 produced
115 embryo mortality in which the allantoic fluid was confirmed to be positive using the HA assay
116 (**Supplementary Table 1**). The estimated *D*-value of HPAI H5N1 virus in raw clinical milk stored
117 at 20°C was 2.23 days. At 30°C, we detected a rapid decrease in infectious HPAI H5N1 virus titers
118 in raw clinical milk, which dropped from 5.8 ± 0.43 log $TCID_{50}$/mL on day 0 to 1.63 ± 0.29 log
119 $TCID_{50}$/mL on day 5 resulting in about 4-log reduction in infectious virus titers **(Fig. 1c)**. Milk
120 samples collected on days 6 and onward were toxic to cell culture and were inoculated into ECEs.
121 On day 6, two out of three milk samples produced embryo mortality, and the allantoic fluids were
122 HA positive, whereas all three milk samples collected on day 7 produced embryo mortality and
123 the allantoic fluids were HA positive. **(Fig. 1c, Supplementary Table 1)**. The estimated *D*-value
124 of HPAI H5N1 virus in raw clinical milk stored at 30°C was 1.21 days. At 37°C, we observed a
125 drastic drop in HPAI H5N1 virus titers, with a 3-log reduction in virus infectivity within the first
126 day of incubation, decreasing from 5.8 ± 0.43 log $TCID_{50}$/mL on day 0 to 2.88 ± 0.14 log
127 $TCID_{50}$/mL on day 1 **(Fig. 1d)**. On day 2 and onward, no infectious virus was detected in cell
128 culture. However, virus isolation in ECEs was successful in one out of three milk samples collected
129 on day 2 **(Fig. 1d, Supplementary Table 1)**. The *D*-value of HPAI H5N1 virus in raw clinical
130 milk stored at 37°C was not calculated as virus was isolated from only two time points.

131 To confirm the results obtained in raw clinical milk positive for HPAI H5N1, we tested the
132 decay of the virus in raw normal milk spiked with the virus. As shown in Fig. 1e, 1f, 1g, and 1h,
133 similar rate and time of virus infectivity decay were observed at the temperatures of 20°C, 30°C
134 and 37°C. At 4°C, the decline in HPAI H5N1 titers in spiked raw milk samples (Fig. 1e) was more
135 linear than what we observed in clinical raw milk samples infected with the virus (Fig. 1a).
136 However, similar to the results obtained in raw clinical milk samples infectious virus was still
137 detected on raw spiked milk samples on days 49 and 56 of incubation. These results show that
138 HPAI H5N1 virus presents long term stability in raw milk stored at 4°C, with viral titers decreasing
139 rapidly at temperatures above 30°C.

140 **Thermal treatment of raw HPAI H5N1 virus positive milk following thermization conditions**
141 **and FDA approved pasteurization results in efficient virus inactivation.** Initially, we evaluated

142 the efficacy of thermization at 60°C for 10 min and the FDA approved pasteurization conditions
143 (63°C for 30 min and 72°C for 15 sec), on the inactivation of HPAI H5N1 virus in raw milk
144 collected from clinically affected cows or in raw milk spiked with HPAI H5N1 virus utilizing a
145 thermocycler. Following heat treatment, milk samples were subjected to virus inoculation and
146 titrations in Cal-1 cells and ECEs. While robust virus replication was detected by
147 immunofluorescence (IFA) in cells inoculated with control non-heat-treated milk samples, no
148 evidence of virus infectivity in raw milk subjected to heat treatment at 60°C for 10 min and at
149 63°C for 30 min or 72°C for 15 sec was observed in Cal-1 cells (**Fig. 2a**).

150 We then quantified viral RNA by qRT-PCR and determined infectious virus titers in control
151 and heat-treated milk samples using cell culture and ECEs. Similar levels of viral RNA were
152 detected in control and in heat-treated milk samples (**Fig. 2b**). Importantly, viral titrations revealed
153 that non-heat-treated raw milk clinical samples had a virus titer of 6.47 ± 0.29 log $TCID_{50}$/mL and
154 7.47 ± 0.29 log EID_{50}/mL, while no infectious virus was detected after heat treatment of the raw
155 milk clinical samples, in cell culture or ECEs, respectively (**Fig. 2c-2d**).

156 We further assessed the thermal inactivation of HPAI H5N1 in normal raw milk spiked
157 (1:10 ratio) with raw milk positive for HPAI H5N1 collected from clinically affected cows. We
158 followed the same heat treatment conditions described above utilizing a thermocycler. Similar to
159 the results observed in raw milk from clinical cows, robust HPAI H5N1 virus replication was
160 observed in Cal-1 cells inoculated with non-heat-treated spiked milk samples, whereas no virus
161 replication was observed after heat treatment at 60°C for 10 min or at 63°C for 30 min or 72°C for
162 15 sec (**Fig. 2e**). While the viral RNA levels remained stable after heat treatment (**Fig. 2f**),
163 complete virus inactivation was observed in spiked milk samples following heat treatment (**Fig.
164 2g-2h**). These results suggest that thermization at 60°C for 10 min and pasteurization at 63°C for
165 30 min or 72°C for 15 sec efficiently led to HPAI H5N1 virus inactivation in raw milk.

166 **Evaluation of thermization conditions on HPAI H5N1 virus inactivation in raw milk.** We next
167 evaluated the efficacy of three additional thermization conditions on HPAI H5N1 virus inactivation
168 in raw milk. For this, raw normal milk was spiked with a HPAI H5N1 virus stock amplified in Cal-
169 1 cells (1:20 ratio) and subjected to thermal treatment at 50°C for 10 min, 63°C for 22 sec and
170 69°C for 22 sec utilizing a thermocycler. Inoculation of the control and heat-treated samples in
171 Cal-1 cells revealed robust HPAI H5N1 virus replication in control non-treated raw spiked milk

172 samples. Additionally, HPAI H5N1 virus replication was detected by IFA in cells inoculated with
173 raw spiked milk heat treated at 50°C for 10 min, however, the proportion of infected cells and the
174 intensity of staining was lower compared to the control non-treated raw milk samples (**Fig. 3a**).
175 No virus replication was detected in cells inoculated with raw HPAI-spiked milk samples heated
176 at 63°C for 22 sec and 69°C for 22 sec, suggesting efficient virus inactivation under these thermal
177 conditions.

178 Quantification of HPAI H5N1 virus RNA by qRT-PCR revealed similar viral RNA levels
179 in control non-treated and all three heat-treated raw spiked milk samples (**Fig. 3b**). Viral titrations
180 have shown that heat treatment of raw HPAI-spiked milk at 50°C for 10 min led to 1.5-2 log
181 reduction in infectious virus titers, which decreased from 4.94 ± 0.21 to 3.19 ± 0.81 log $TCID_{50}$/mL
182 or 4.87 ± 0.13 to 2.87 ± 0.35 log EID_{50}/mL after heat treatment (**Fig. 3c-d**). No infectious virus
183 was detected in raw HPAI-spiked milk samples heat treated at 63°C for 22 sec and 69°C for 22
184 sec. These results show that while thermization at 50°C for 10 min does not completely inactivate
185 HPAI H5N1 virus in raw milk, higher temperatures of 63 and 69°C efficiently and rapidly
186 inactivate the virus in raw milk.

187 **Validation of thermal inactivation of HPAI H5N1 virus in raw milk using a submerged coil**
188 **system.** To more closely simulate the thermal treatment conditions (e.g. circulating hot water heat
189 source and rapid ramp up temperatures) to which raw fluid milk is subjected to during commercial
190 pasteurization in the dairy industry, we developed a submerged coil heating system to heat treat
191 milk samples (**Fig. 4a**). The system holds 15 mL of milk within a coil and is heated with a
192 circulating water bath. The submerged coil heating system is furnished with four thermal sensors
193 that are connected to a data logger to continuously monitor and record the milk temperature as it
194 flows through four segments of the coil (**Fig. 4a**). The first coil segment (capacity of 4.2 mL)
195 contains two portions, including one external coil component carrying the sample injection port
196 and a temperature sensor (T1) (**Fig. 4a**) to monitor the temperature of the milk at the injection port
197 (**Fig. 4b**); and one submerged coil component (**Fig. 4a**) which preheats the milk sample to the
198 desired temperature within 7-10 sec (**Fig. 4b**) as it flows through this portion of the coil. The
199 temperature of the milk that flows through the pre-heating coil segment is recorded by thermal
200 sensor T2 (**Fig. 4a** and **4b**). The second (capacity 5.5 mL) and third (capacity 5.7 mL) coil
201 segments are completely submerged in water and hold the sample at the target temperature for the

202 intended time. The thermal sensor T3 monitors the milk temperature in the holding coil segment
203 (**Fig. 4a** and **4b**). The fourth coil segment (capacity of 1.5 mL) consists of the exit loop which
204 dispenses the milk in a collection tube after the heat treatment. Cooling of the milk after thermal
205 treatment was achieved by placing the collection tubes on ice for 10 min, immediately after sample
206 collection.

207 To validate the results obtained with the thermocycler, we evaluated the thermal
208 inactivation of HPAI in raw milk using the submerged coil system following the FDA approved
209 pasteurization conditions at 63°C for 30 min and 72°C for 15 s, and four thermization conditions
210 at 50°C for 10 min, 60°C for 10 min, 63°C for 22s and 69°C for 22s. For each thermal treatment
211 condition, 15 mL of raw milk spiked with HPAI H5N1 virus (1:20 ratio) were injected into the
212 submerged coil heating system and subjected to heat treatment at the desired temperatures and
213 times. Temperature monitoring as recorded by the thermal sensors data loggers following each
214 treatment confirmed that the milk samples were subjected to the heat treatment at the target
215 temperatures (**Fig. 4b, Extended Data Fig. 2**). Initially, virus infectivity was monitored by
216 inoculation of control and thermal treated spiked milk samples in Cal-1 cells. As shown in **Fig. 4c**,
217 virus replication was detected in control non-treated samples and in milk samples subjected to heat
218 treatment at 50°C for 10 min, as evidenced by positive viral NP staining in inoculated cells. Similar
219 to the results obtained with the experiments conducted in the thermocycler, a lower number of NP
220 positive cells were observed in cells inoculated with milk samples subjected to heat treatment at
221 50°C for 10 min, when compared to control non-treated samples (**Fig. 4c**). No evidence of virus
222 replication was detected in HPAI-spiked milk samples heat treated at 60°C for 10 min, 63°C for
223 22 s, 63°C for 30 min, 69°C for 22 s and 72°C for 15 s.

224 Next, we quantified viral RNA and infectious virus in all control and thermal treated
225 samples. The qRT-PCR results revealed comparable levels of viral RNA in both heat-treated and
226 non-treated milk samples (**Fig. 4d**). Virus quantifications in Cal-1 cells or ECEs demonstrated viral
227 titers of 4.66 ± 0.19 log $TCID_{50}$/mL and 4.54 ± 0.03 log EID_{50}/mL in untreated milk samples,
228 which decreased by one log in milk samples heat treated at 50°C for 10 min, with titers of 3.69 ±
229 0.38 log $TCID_{50}$/mL and 3.5 log EID_{50}/mL (**Fig. 4e-f**). Notably, no infectious virus titers were
230 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
231 22 s and 72°C for 15 s. These results corroborate the findings of our studies using a thermocycler

232 and demonstrate the efficacy of a broad spectrum of thermal treatment conditions on inactivation
233 of HPAI H5N1 virus in raw milk.

234 **Kinetics of HPAI H5N1 virus inactivation by thermal treatment in raw milk.** We evaluated
235 the kinetics of thermal inactivation of HPAI H5N1 virus in raw milk at different temperatures over
236 time using the submerged coil system. For this, raw HPAI-spiked milk was subjected to the thermal
237 treatment and samples were collected at different time points as follows: 50°C - 5, 10, 15 20, 25,
238 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,
239 10, 12, and 15 sec. Viral RNA and infectious virus were quantified by qRT-PCR and virus titrations
240 (cells and ECEs), respectively. Viral RNA levels detected in untreated and heat-treated milk
241 samples were comparable in all temperatures evaluated in the study (**Fig. 5a, d, g, j**). At 50°C, we
242 observed a gradual decrease in infectious virus titers from 4.88 ± 0.14 to 2.30 ± 0.25 log
243 $TCID_{50}$/mL within 25 min heat treatment with virus titers falling under the titration detection limit
244 between the 25- and 30-min time point (**Fig. 5b**). Similar results were observed in ECEs with viral
245 virus titers decreasing from 4.58 ± 0.07 to 1.17 ± 1.01 log EID_{50}/mL within 25 min (**Fig. 5c**). Of
246 note, one out of three milk samples heat treated at 50°C for 30 min showed embryo mortality and
247 HA activity, indicating the presence of low amounts of infectious virus in this sample. The *D*-value
248 of HPAI H5N1 at 50°C was estimated at 10.11 min. Notably, rapid virus inactivation of HPAI
249 H5N1 was observed in raw milk samples heat treated at 60°C (**Fig. 5e, 5f**), 63°C (Fig. **5h, 5i**) and
250 72°C (**Fig. 5k, 5l**), with heating for 5 sec leading to complete virus inactivation at these
251 temperatures. These results demonstrate that HPAI H5N1 virus is stable for at least 30 min at 50°C
252 in raw milk. Importantly, however, efficient and rapid inactivation (within five seconds) of HPAI
253 H5N1 virus is achieved in raw milk heat treated at temperatures of 60°C or above.

254 **Determination of thermal resistance constant *z*-value.** We determined the thermal resistance
255 constant *z*-value of HPAI H5N1 in raw milk. For this, raw HPAI-spiked milk was heat treated at
256 50°C, 52°C, 54°C, 56°C, 58°C and 60°C using the submerged coil system (**Extended Data Fig.**
257 **3**) and samples were collected at 5 min intervals for up to 30 min. Infectious virus titers were
258 quantified by virus titration in ECEs. At 50°C, we observed a gradual decrease in virus titers from
259 7.59 ± 0.2 log EID_{50}/mL to 3.17 ± 0.58 log EID_{50}/mL within 30 min of heat treatment (**Fig. 6b**).
260 At 52°C, there was a 4.5-log reduction in virus titers within 25 min of heat treatment from 7.59 ±
261 0.2 log EID_{50}/mL to 1.44 ± 1.29 log EID_{50}/mL with complete virus inactivation after 30 min of

262 heat treatment. At 54°C, there was a rapid decrease (approximately 6-log) in virus titers from 7.59
263 ± 0.2 log EID_{50}/mL to 1.13 ± 0.96 log EID_{50}/mL within 10 min of heat treatment with complete
264 virus inactivation following 15 min heat treatment. At 56°C and 58°C, virus titers dropped rapidly
265 (6-log) within 5 min of heat treatment from 7.59 ± 0.2 log EID_{50}/mL to 1.67 ± 1.44 and 1.13 ±
266 0.96 log EID_{50}/mL, respectively and were undetected afterwards. Of note, rapid virus inactivation
267 was observed at 60°C as no infectious virus was detected following 5 min heat treatment (**Fig. 6b**).
268 The D-values at 50°C, 52°C and 54°C were estimated at 7.19 min, 4.74 min and 2.04 min,
269 respectively. The D-values from the temperatures of 56°C, 58°C and 60°C couldn't be calculated
270 as there were only 1-2 data points. Based on these results we estimated the thermal resistant
271 constant z-value of HPAI H5N1 virus in raw milk to be 9.93°C.

272 **Discussion**

273 The high tropism of HPAI H5N1 virus and its replication in milk secreting epithelial cells of the
274 mammary gland in dairy cows, lead to high viral loads and shedding (from $10^{4.4}$ to up $10^{8.8}$
275 $TCID_{50}.mL^{-1}$) in milk[6,8,16]. Despite the widespread use of milk pasteurization by the dairy industry,
276 the high viral loads detected in milk from infected cows raised major public health concerns.
277 Although it has been shown that both high (HPAI)- and low pathogenic avian influenza (LPAI)
278 viruses can be inactivated by pasteurization of various poultry products including fat-free egg
279 products, allantoic fluid,plasma[17], and milk byproduct concentrated lactose[18], in the beginning of
280 the dairy outbreak there was no data demonstrating the efficacy of pasteurization on HPAI H5N1
281 virus in dairy products. Initial laboratory studies evaluating inactivation of HPAI in raw milk
282 revealed that the most used FDA approved milk pasteurization condition (72°C for 15 sec)
283 markedly reduced viral loads in raw milk, however, low amounts of infectious virus were still
284 detected in heat treated samples[13,19]. Here we evaluated the stability and determined the decay of
285 HPAI H5N1 virus in raw milk stored at different temperatures over time and investigated the
286 efficacy and kinetics of inactivation of HPAI H5N1 virus following the two FDA approved
287 pasteurization- as well as various thermization (subpasteurization) conditions widely used by the
288 dairy industry.

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

292 in these samples. These results corroborate findings of a recent study in which infectious HPAI
293 H5N1 was recovered for up to 5 weeks from milk samples spiked with the virus[13], highlighting
294 the potential public health risk posed by consumption of raw milk and other raw milk derived
295 products such as raw milk cheeses which must be aged for 60 days at 4°C[20] prior to human
296 consumption. Survival of HPAI H5N1 virus in raw milk incubated at 4°C for 56 days with residual
297 titers of about 3.5-3.8 log $EID_{50}.ml^{-1}$ as shown here (**Fig. 1a**), underscore the need for further
298 studies to determine whether the aging process efficiently inactivates HPAI H5N1 virus in raw
299 milk cheeses. The decay studies performed here provide valuable information regarding the
300 sensitivity of HPAI H5N1 to higher temperatures. Incubation of raw clinical milk from infected
301 cows as well as HPAI-spiked raw normal milk at 20°C resulted in inactivation of the virus within
302 21 days (3 weeks), whereas rapid virus decay was observed at 30°C (6 days, <1 week) and 37°C
303 (2 days) in spiked milk samples. Therefore, thermal treatment of milk from infected cows to
304 temperatures between 30-37°C, could potentially be utilized to inactivate HPAI H5N1 virus in raw
305 milk prior to its disposal in affected farms. This would minimize the risk of environmental
306 contamination and further virus spread.

307 Although the grade A pasteurized milk ordinance (PMO)[21] requires that milk from sick
308 animals is segregated on the farm, FDA and USDA found PCR positive shelf milk samples[12],
309 indicating that the virus found its way into bulk raw milk making it into grocery stores. Importantly,
310 follow up testing of these samples to determine virus infectivity have shown that no infectious
311 virus was present in these PCR positive samples[12], which is likely a result of pasteurization. While
312 early laboratory studies have shown that heat treatment of HPAI H5N1 spiked milk samples to
313 conditions that mimic FDA approved pasteurization conditions (63°C for 30 min and 72°C for 15
314 sec) results in drastic reduction in viral titers (about 4.5-log EID_{50}), residual virus infectivity was
315 still detected when these samples were subjected to the thermal treatment at 72°C for 15 sec[13,19].
316 Results here, however, using raw milk from HPAI infected cows or raw milk spiked with HPAI
317 H5N1 virus, show that the two FDA approved pasteurization conditions efficiently inactivated the
318 virus resulting in up to 7.3 log EID_{50} reduction in virus infectivity. The discrepancies between our
319 study and the two previous studies could be caused by differences in heat treatment methods or
320 equipment. While the studies that showed residual infectivity of the virus were conducted only
321 using thermocyclers to heat inactivate the virus, we used the thermocycler method and a
322 submerged coil heating system which can record the sample temperature as it flows through and

323 is incubated within the heating coils. This capability unequivocally demonstrates the efficiency of
324 pasteurization and several thermization temperatures in inactivating HPAI H5N1 virus in raw milk.
325 Notably, our findings using this controlled heating system corroborate and strengthens results of a
326 study conducted by USDA and FDA which showed that HTST in a lab-scale continuous flow
327 pasteurizer efficiently inactivated HPAI H5N1 in raw milk[22]. Together, these findings demonstrate
328 that pasteurization is highly effective in reducing the load of HPAI H5N1 virus in milk.

329 We extended our thermal inactivation studies to include four thermization conditions that
330 are frequently used in dairy industry for production of raw milk cheeses[14,15]. Three out of four
331 thermization conditions tested including 60°C for 10 min, 63°C for 22 s and 69°C for 22 s resulted
332 in inactivation of HPAI in raw milk. Thermization of raw milk at 50°C for 10 min, however, only
333 partially reduced HPAI H5N1 virus titers (0.83-2.42 log EID_{50} reduction). These results
334 demonstrating lack of inactivation of the virus in raw milk at 50°C and complete inactivation at
335 60°C, led us to investigate virus decay in temperatures between 50-60°C to determine the thermal
336 resistant constant *z*-value of HPAI H5N1 in raw spiked milk. Our results indicate that HPAI H5N1
337 TX2/24 virus had *z*-values of 9.93-10.33°C in raw milk. which is slightly higher than z-values
338 (4.58-4.69°C) of a Korean H5N1 virus in chicken meat[23]. Together these results demonstrate that
339 HPAI H5N1 is readily inactivated in raw milk subjected to thermal treatment at temperatures on
340 and above 60°C, but even temperatures above 54°C efficiently inactivate the virus within 15 min
341 of treatment. This was confirmed by our inactivation kinetics studies. Based on our calculated *D*-
342 value at 50°C, it would take 50.55 minutes to achieve a 5-log reduction in HPAI H5N1 loads in
343 contaminated raw milk. In FDA's survey of bulk milk tanks, they observed an average 3.5 log
344 EID_{50} load, with a range of 1.3 to 6.3 log EID_{50}[22]. A 10°C increase in the treatment temperature to
345 60°C resulted in rapid virus inactivation within 5 seconds of treatment, and while the inactivation
346 was too fast to capture shorter-time intervals, the approximated *D*-value is 1.04s (1/ (4.8 log
347 EID_{50}/5)), assuming a linear decay over 5 seconds, highlighting the efficacy of thermal treatment
348 in ensuring safety of milk and other dairy products derived from pasteurized milk. Additionally,
349 several of the thermization conditions tested in our controlled experimental settings proved
350 effective against HPAI H5N1, suggesting the use of thermized milk treated at 60°C and above for
351 15-20 seconds to produce cheeses would reduce the risk of human exposure to products with
352 infectious HPAI H5N1.

353 In summary, our study provides a comprehensive overview of the thermal inactivation
354 spectrum of HPAI H5N1 virus in raw milk, demonstrating the efficacy of thermal treatment
355 including thermization and pasteurization conditions on inactivation of HPAI H5N1 virus in raw
356 milk.

357 **Materials and Methods**

358 **Cells and virus**

359 Bovine uterine epithelial cells (Cal-1, developed in house at the Virology Laboratory at AHDC)
360 and Madin-Darby canine kidney (MDCK) cells were cultured in minimal essential medium (MEM,
361 Corning Inc., Corning, NY) supplemented with 10% fetal bovine serum (FBS) and penicillin-
362 streptomycin (ThermoFisher Scientific, Waltham, MA; 10 $U.mL^{-1}$ and 100 $\mu g.mL^{-1}$, respectively).
363 Primary chicken embryo fibroblast (CEF) was cultured in Medium 199, Earle's Salts (Gibco, U.S.)
364 supplemented with 10% FBS and anti-anti (Gibco, U.S.). Primary bovine mammary epithelial cells
365 (bMEC) were grown in William's Medium E supplemented with 10% FBS, L-glutamine (Gibco,
366 U.S.), 1X Insulin-Transferrin-Selenium (ITS) (Gibco, U.S.) and 10 ng/mL Epithelial Growth
367 Factor (EGF) (VWR - Life Cell Technology, U.S.). The HPAI H5N1 TX2/24
368 (A/Cattle/Texas/063224-24-1/2024, genotype B3.13, GISAID accession number:
369 EPI_ISL_19155861) isolated from the milk of infected dairy cattle in Texas, USA[6] was used in
370 the decay and pasteurization studies. The virus stock was propagated and titrated in Cal-1 cells.

371 **Biosafety and biosecurity**

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

374 **Viral growth kinetics**

375 Viral growth kinetics were performed using primary bovine mammary epithelial cells (bMEC),
376 bovine uterine epithelial cells (Cal-1), primary chicken embryo fibroblast (CEF) and Madin-Darby
377 canine kidney (MDCK) cells. Cells were seeded in 12-well plates ($1.2x10^5$ cells/mL) for 24 hours
378 until they reached 80-90% confluence. Cells were then infected with the HPAI H5N1 TX2/24 virus
379 at the multiplicity of infection (MOI) of 0.1 and incubated at 4ºC for 1 hour for virus adsorption.

380 The inoculum was then replaced with 1 mL of complete growth media and incubated at 37ºC. Cells
381 and supernatant were harvested at 4-, 8-, 12-, 24-, 48- and 72-hours post-inoculation and stored at
382 -80ºC. Time point 0 was an aliquot of virus inoculum stored at -80ºC as soon as inoculation was
383 completed. Virus titers were determined in Cal-1 cells at each time point using end-point dilutions
384 and the Spearman and Karber's method and expressed as $TCID_{50}.mL^{-1}$.

385 **Milk samples**

386 Normal raw milk samples were obtained from Teaching Dairy at Cornell University and used as
387 negative control as well as the matrix to be spiked with HPAI H5N1 virus during heat treatment
388 studies. Raw clinical milk samples used in the decay and thermal treatment studies were collected
389 by field veterinarians from a clinically affected dairy farm in Texas, United States [6]. Pool milk
390 samples from seven HPAI infected dairy farms were used in decay study (Fig. 1a-b) and heat
391 treatment studies (Fig. 2).

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

393 For thermal decay studies, raw clinical milk samples from HPAI infected dairy cows and/or HPAI-
394 spiked raw milk samples were used. For raw clinical milk samples, IAV M RT-PCR positive milk
395 samples from seven HPAI infected dairy cows from Texas were used. Three pools of milk samples
396 from different farms were prepared to run three independent decay experiments. The raw clinical
397 milk samples (1 mL aliquot) were incubated at 4°C, 20°C, 30°C and 37°C. For HPAI-spiked
398 samples, raw normal milk samples obtained from Cornell Teaching Dairy were spiked with HPAI
399 H5N1 TX2/24 (stock titer $2x10^7$ log $TCID_{50}$/mL) at 1:20 dilution to achieve a final titer of 6 log
400 $TCID_{50}$/mL. The HPAI-spiked milk samples (1 mL aliquot) were then incubated at 4°C, 20°C,
401 30°C and 37°C. For all temperatures, sequential samples were collected on days 0, 1, 2, 3, 4, 5, 6,
402 7, 14, 21, 28, 35, 42, 49 and 56 and stored at -80°C for virus quantifications.

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

404 Initially, the thermal treatment of raw milk to assess inactivation of HPAI H5N1 was performed
405 using a thermocycler. Both pooled raw clinical milk samples from HPAI infected cows and HPAI-
406 spiked raw milk samples were used. For HPAI-spiked samples, raw normal milk obtained from the

407 Cornell Teaching Dairy were spiked with HPAI H5N1 TX2/24 (stock titer $2x10^7$ log $TCID_{50}$/mL)
408 at 1:20 ratio to achieve a target titer of 6 log $TCID_{50}$/mL. For heat treatment, we used combinations
409 of six different temperatures and holding times that are used for thermal processing of milk and
410 milk products in dairy industry. We tested two pasteurization conditions, low temperature long
411 time (LTLT) at 63°C 30 min and high temperature short time (HTST) at 72°C 15 s as well as four
412 thermization conditions of 50°C for 10 min, 60°C for 10 min, 63°C for 22 sec and 69°C for 22 sec.
413 For heat treatment, 100 µL/tube of raw clinical milk or HPAI-spiked raw normal milk samples
414 were added in 8-tube PCR strip. A total of 4 strips (3.2 mL milk) were used per replicate. Following
415 heat treatment in a thermocycler (BioRad T100 Thermal Cycler), milk samples were chilled
416 immediately on ice for 10 min. The milk samples were pooled, three aliquots of 1 ml volume were
417 prepared for each replicate and stored at -80°C until further testing.

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

419 To more closely simulate pasteurization conditions, we developed a submerged coil heating
420 system. The stainless-steel coil tubing (FITOK 316/316L Stainless Steel Seamless Tubing, 1/8"
421 OD × 0.028" Wall, Coil) system has four segments (Fig. 4a) that are furnished with four
422 temperature sensors (Quick Disconnect Thermocouples with Miniature Connectors, Omega
423 Engineering Inc., USA) at each segment. The first segment consists of two injection ports for
424 sample and water and is partly submerged into a water bath. The second and third loops are
425 completely submerged in water and hold the desired temperature immediately after the sample
426 injection. The fourth segment is the exit loop for sample collection and is partly submerged into
427 the water. The thermal sensors are connected to a data logger (HH378, Data Logger/K, J, E, T,
428 Type Thermometer, Omega Engineering Inc, USA) and temperatures are recorded throughout the
429 heat treatment process. Temperature data from the datalogger were retrieved by using Se378
430 software (Omega Engineering Inc, USA). A circulating water bath (Cole-Parmer EW-16101-89:
431 Unheated Water Bath, Fisher Scientific) furnished with Immersion Circulators (VWR) was used
432 to heat the coils. About 15 mL of milk samples were injected into the sample port by using a 20
433 mL syringe. After incubation at the desired temperature and time, milk samples (1-5 mL) were
434 pushed out of the coil system by injecting sterile water in the system. Each milk sample was
435 collected into a sterile tube. Immediately after collection, the milk samples were chilled on ice for
436 10 min, aliquoted and stored at -80°C. After each round, the coil was cleaned by flushing the coil

437 with a 1.5% w/v solution of alkaline detergent (Alcanox, Sigma-Aldrich) followed by 1% w/v
438 solution of acid detergent (Citranox, Alconox) and finally passing several coil-full volumes of
439 sterile water and by passing the air several times.

440 **RNA extraction and RT-PCR**

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

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

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

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

461 For virus titration in eggs, 9-days-old embryonated chicken eggs were used. Serial 10-fold
462 dilutions of milk samples were prepared in phosphate buffered saline (PBS) supplemented with
463 antibiotic-antimycotic (anti-anti 100X, Thermo Fisher Scientific, USA) at 2:1 ratio. The eggs were
464 candled to mark the air sac on the shell, sanitized with 70% ethanol and a hole was drilled in the
465 eggshell. One hundred µL of the diluted milk sample was injected in quadruplicate into the chorio-

466 allantoic sac route and sealed with glue. Eggs were candled daily for four days, and dead embryos
467 were chilled overnight before collection. After 4 days post-inoculation, all surviving embryos were
468 chilled for 24 hours, and allantoic fluids were collected. All allantoic fluids were tested by
469 hemagglutination assay (HA) using 0.5% chicken RBC. Finally, the 50% egg infectious dose
470 (EID_{50}) was calculated by the Reed and Muench method.

471 **Hemagglutination assay (HA)**

472 For the HA, 100 µL allantoic fluid was taken into the wells of 1st row of a 96-well U-bottom plate.
473 50 µL of PBS was added to the wells of 2nd, 3rd and 4th rows. Three 2-fold dilutions (1:2, 1:4 and
474 1:8) of the allantoic fluids were prepared in PBS. Then, 50 µL of 0.5% chicken red blood cells
475 (RBC) were added to each well and incubated at room temperature for 30 min. Lack of RBC button
476 formation indicates positive HA reaction.

477 **Determination of D- and Z-values**

478 The thermal inactivation kinetics of HPAI H5N1 in raw milk were calculated based on the decimal
479 reduction time (*D*-value) and the thermal resistance constant (z-value). The *D*-value is defined as
480 the time required at a specific temperature to achieve a one-logarithm reduction in viral titer. This
481 was determined by plotting the logarithm (base 10) of the infectious viral titer against time for
482 each tested temperature. The *D*-value was then calculated as the negative inverse of the slope of
483 the resulting plot, with the line of best fit for the survivor curves established through regression
484 analysis. To calculate the z-value of HPAI H5N1, which indicates the temperature change needed
485 to produce a tenfold change in the *D*-value, HPAI-spiked milk samples were heat treated at 50°C,
486 52°C, 54°C, 56°C, 58°C and 60°C with the submerged coil heating system and sequential milk
487 samples were collected at every 5 min interval up to 30 min. Virus titers were determined by using
488 embryonated chicken eggs ($EID_{50}.mL^{-1}$) and *D*-values were calculated as above. The linear
489 regression of the logarithm (base 10, Log) of the *D*-values was performed against their
490 corresponding heating temperatures. The absolute values of the inverse of the slope were used to
491 calculate the *z*-values.

492 **Statistical analysis**

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

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Data availability

All data pertaining to this study are presented in the paper, in the Extended Data and are available from the corresponding author upon request. Source data are provided with this paper.

Author contributions

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

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567

568 **Figure legends**

569 **Fig. 1.** Kinetics HPAI H5N1 virus decay in milk. Decay of HPAI H5N1 virus in raw clinical milk
570 collected from HPAI infected cows incubated at 4°C (**a**), 20°C (**b**), 30°C (**c**) and 37°C (**d**) for 56
571 days. Decay of HPAI H5N1 virus in HPAI-spiked (1:20 ratio) raw normal milk and incubated at
572 4°C (**e**), 20°C (**f**), 30°C (**g**) and 37°C (**h**) for 56 days. The virus titers were determined by end point
573 titrations in Cal-1 cells (Log $TCID_{50}.mL^{-1}$). Data represents observations (dots) from three
574 replicates (n=3) overlayed with the mean (horizontal line) and ± SEM (whiskers) from 1(a) or 3
575 (b-h) independent experiments.

576 **Fig. 2.** Effect of low temperature thermization and FDA approved pasteurization conditions on
577 HPAI H5N1 infectivity in raw milk. **a-d.** Thermal inactivation of HPAI H5N1 virus in raw clinical
578 milk samples collected from HPAI infected cows. **a.** Immunofluorescence (IFA) images of Cal-1
579 cells inoculated with control (normal and clinical) or heat-treated clinical milk samples. Raw
580 clinical milk samples were collected from HPAI H5N1 infected cows and heat treated at the
581 indicated temperatures and times utilizing a thermocycler. **b.** HPAI H5N1 viral RNA loads in
582 control or heat-treated raw clinical milk samples as determined by qRT-PCR (n = 3). **c.** HPAI H5N1
583 virus titrations in Cal-1 cells (n = 3). **d.** HPAI H5N1 virus titrations in embryonated chicken eggs
584 (n = 3). **e-h.** Thermal inactivation of HPAI H5N1 virus in raw normal milk spiked (1:10 ratio) with
585 milk samples collected from HPAI infected cows using a thermocycler. **e.** IFA images of Cal-1

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

595 **Fig. 3.** Evaluation of thermization conditions on inactivation of HPAI H5N1 virus in raw milk.
596 Thermal inactivation of HPAI H5N1 virus in raw normal milk spiked (1:20 ratio) with HPAI H5N1
597 using a thermocycler. **a.** IFA images of Cal-1 cells inoculated with control or heat-treated HPAI-
598 spiked raw normal milk samples. **b.** HPAI H5N1 viral RNA loads in control or heat-treated HPAI-
599 spiked raw milk samples as determined by qRT-PCR (n = 3). **c.** HPAI H5N1 virus titrations in Cal-
600 1 cells (n = 3). **d.** HPAI H5N1 virus titrations in embryonated chicken eggs (n = 3). Red
601 fluorescence indicates detection of the viral NP protein. Images are representative of three
602 independent experiments (n = 3). The virus titers were determined by end point dilutions in Cal-1
603 cells and in embryonated chicken eggs and expressed as tissue culture infectious dose 50 per mL
604 ($TCID_{50}.mL^{-1}$) and egg infectious dose 50 per ml ($EID_{50}.mL^{-1}$), respectively. Data represents
605 observations (dots) from three independent experiments (n=3) overlayed with the mean (horizontal
606 line) and ± SEM (whiskers).

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

616 in Cal-1 cells (n = 3). **f.** HPAI H5N1 virus titrations in embryonated chicken eggs (n = 3). Red
617 fluorescence indicates detection of the viral NP protein. Images are representative of three
618 independent experiments (n = 3). The virus titers were determined by end point dilutions in Cal-1
619 cells and in embryonated chicken eggs and expressed as tissue culture infectious dose 50 per mL
620 ($TCID_{50}.mL^{-1}$) and egg infectious dose 50 per ml ($EID_{50}.mL^{-1}$), respectively. Data represents
621 observations (dots) from three independent experiments (n=3) overlayed with the mean (horizontal
622 line) and ± SEM (whiskers).

623 **Fig. 5.** Kinetics of HPAI H5N1 virus inactivation in milk using submerged coil heating system.
624 Raw normal milk was spiked with HPAI H5N1 at 1:20 ratio and heat treated at 50°C (**a-c**), 60°C
625 (**d-f**), 63°C (**g-i**) and 72°C (**j-l**) with a submerged coil heating system. Sequential samples were
626 collected at the indicated time points and analyzed by qRT-PCR and virus titration in cell culture
627 and embryonated chicken eggs. The virus titers were determined by end point titration in Cal-1
628 cells ($TCID_{50}.mL^{-1}$) and embryonated chicken eggs ($EID_{50}.mL^{-1}$). Data represents observations
629 (dots) from three independent experiments (n=3) overlayed with the mean (horizontal line) and ±
630 SEM (whiskers).

631 **Fig. 6.** Thermal resistant constant (*z*-value) of HPAI H5N1 virus in raw milk. Raw normal milk
632 was spiked with HPAI H5N1 at 1:20 ratio and heat treated at 50°C, 52°C, 54°C, 56°C, 58°C and
633 60°C with a submerged coil heating system. Sequential samples were collected at the indicated
634 time points and subjected to virus titration using embryonated chicken eggs ($EID_{50}.mL^{-1}$). The
635 EID_{50} titers were used to calculate the thermal resistant constant *z*-value of HPAI H5N1 in raw
636 milk. Data represents observations (dots) from three independent experiments (n=3) overlayed
637 with the mean (horizontal line) and ± SEM (whiskers).

638 **Extended Data Fig. 1.** Multicycle growth curve of HPAI H5N1 TX2/24. Primary bovine
639 mammary epithelial cells (bMEC), bovine uterine epithelial cells (Cal-1), primary chicken embryo
640 fibroblast (CEF) and Madin-Darby canine kidney (MDCK) cells were infected (MOI 0.1) with HPAI
641 H5N1 TX2/24 and virus titers were determined at indicated time points by limiting dilution method and
642 expressed as $TCID_{50}.mL^{-1}$. Data indicates mean ± SEM, n = 3, three independent experiments.

643 **Extended Data Fig. 2.** Line graph showing temperature changes over time in injected milk
644 samples during heat treatment in the submerged coil heating apparatus at 50°C for 10 min (**a**),

645 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
646 as mean ± SEM of 3 independent experiments.

647 **Extended Data Fig 3**. Line graph showing temperature changes over time in injected milk samples
648 during heat treatment in the submerged coil heating apparatus at 50°C (**a**), 52°C (**b**), 54°C (**c**),
649 56°C (**d**) 58°C (**e**) and 69°C (**f**) over 30 min. Data are presented as mean ± SEM of 3 independent
650 experiments.

651

Supplementary 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		
Raw clinical milk									
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
R4	Toxicity	1:10	1/3	1/3	R4	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
R5	Toxicity	1:10	3/3	3/3	R5	Toxicity	1:10	1/3	1/3
		$1:10^2$	1/3	1/3			$1:10^2$	1/3	1/3
		$1:10^3$	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	1/3	1/3
		$1:10^2$	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	1/3	R2	Toxicity	1:10	1/3	1/3
		$1:10^2$	0/3	0/3			$1:10^2$	1/3	1/3
		$1:10^3$	0/3	0/3			$1:10^3$	0/3	0/3
R3	Toxicity	1:10	3/3	3/3	R3	Toxicity	1:10	1/3	1/3
		$1:10^2$	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	3/3	3/3	R1	Toxicity	1:10	0/3	0/3
		$1:10^2$	2/3	2/3			$1:10^2$	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
HPAI-spiked raw milk									
4°C D49		Dilutions	Death	HA (+)	**4°C D56**		Dilutions	Death	HA (+)

R1	Toxicity	1:10	3/3	3/3	R1	Toxicity	1:10	1/3	1/3
		$1:10^2$	1/3	1/3			$1:10^2$	2/3	2/3
		$1:10^3$	0/3	0/3			$1:10^3$	0/3	0/3
R2	Toxicity	1:10	2/3	2/3	R2	Toxicity	1:10	2/3	2/3
		$1:10^2$	0/3	0/3			$1:10^2$	1/3	1/3
		$1:10^3$	1/3	0/3			$1:10^3$	0/3	0/3
R3	Toxicity	1:10	2/3	2/3	R3	Toxicity	1:10	2/3	2/3
		$1:10^2$	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
20°C D14					**20°C D21**				
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	2/3	0/3
		$1:10^2$	0/3	0/3			$1:10^2$	1/3	0/3
		$1:10^3$	0/3	0/3			$1:10^3$	0/3	0/3
R3	Toxicity	1:10	1/3	1/3	R3	Toxicity	1:10	0/3	0/3
		$1:10^2$	1/3	1/3			$1:10^2$	1/3	1/3
		$1:10^3$	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
R4	Toxicity	1:10	3/3	3/3	R4	Toxicity	1:10	2/3	2/3
		$1:10^2$	1/3	0/3			$1:10^2$	2/3	2/3
		$1:10^3$	1/3	0/3			$1:10^3$	0/3	0/3
R5	Toxicity	1:10	1/3	1/3	R5	Toxicity	1:10	1/3	1/3
		$1:10^2$	1/3	0/3			$1:10^2$	0/3	0/3
		$1:10^3$	1/3	1/3			$1:10^3$	2/3	2/3
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
R4	Toxicity	1:10	1/3	1/3	R4	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
R5	Toxicity	1:10	0/3	0/3	R5	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.

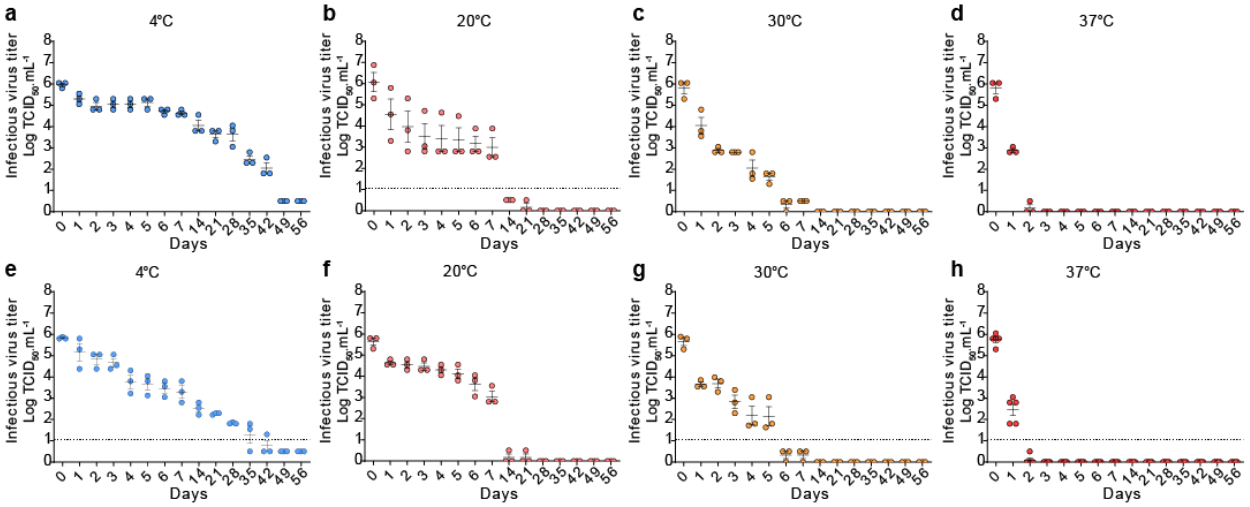


Fig. 1. Kinetics HPAI H5N1 virus decay in milk. Decay of HPAI H5N1 virus in raw clinical milk collected from HPAI infected cows incubated at 4°C (**a**), 20°C (**b**), 30°C (**c**) and 37°C (**d**) for 56 days. Decay of HPAI H5N1 virus in HPAI-spiked (1:20 ratio) raw normal milk and incubated at 4°C (**e**), 20°C (**f**), 30°C (**g**) and 37°C (**h**) 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) from 1(a) or 3 (b-h) independent experiments.

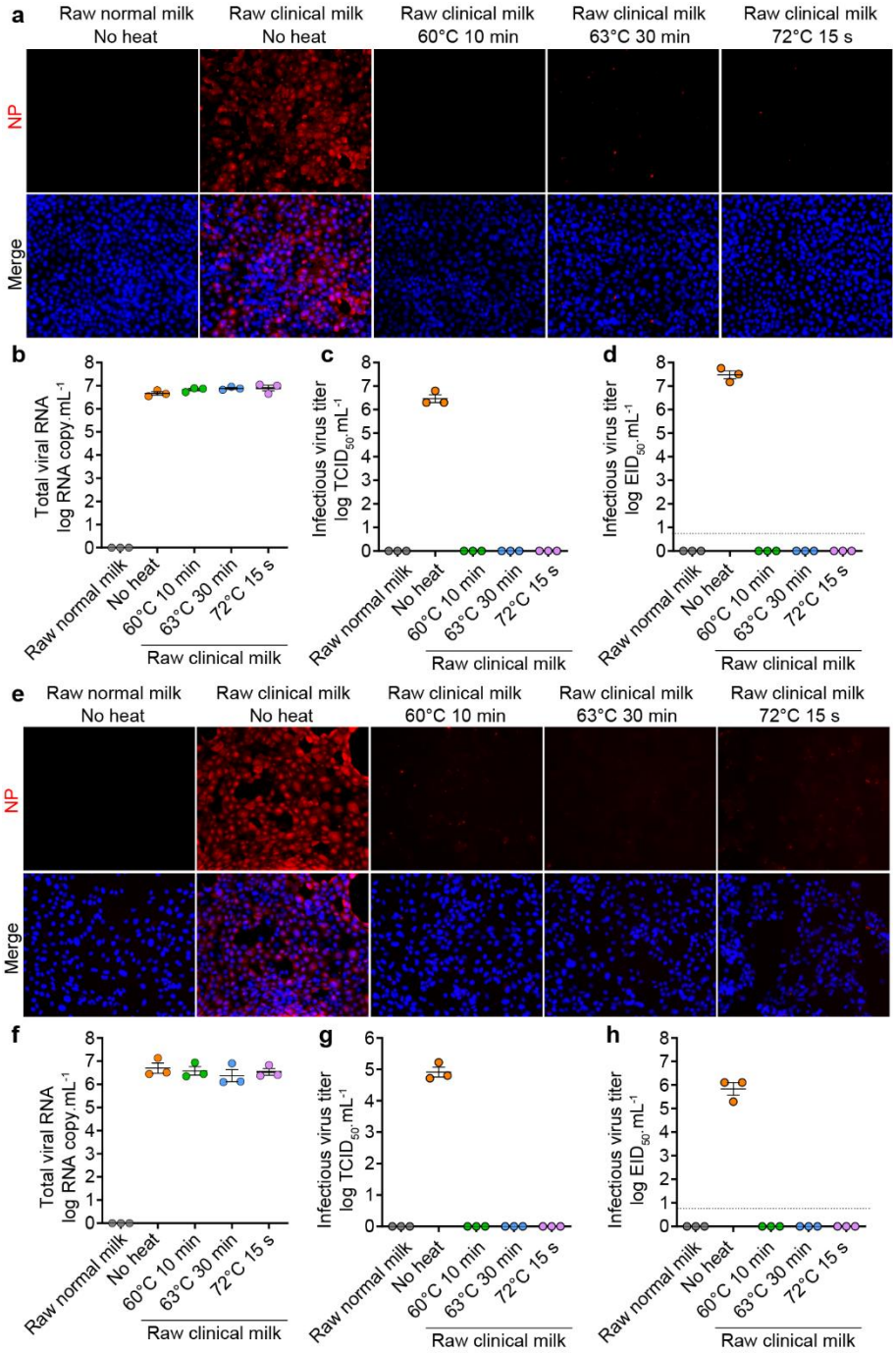


Fig. 2. Effect of low temperature thermization and FDA approved pasteurization conditions on HPAI H5N1 infectivity in raw milk. **a-d.** Thermal inactivation of HPAI H5N1 virus in raw clinical milk samples collected from HPAI infected cows. **a.** Immunofluorescence (IFA) images of Cal-1 cells inoculated 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).

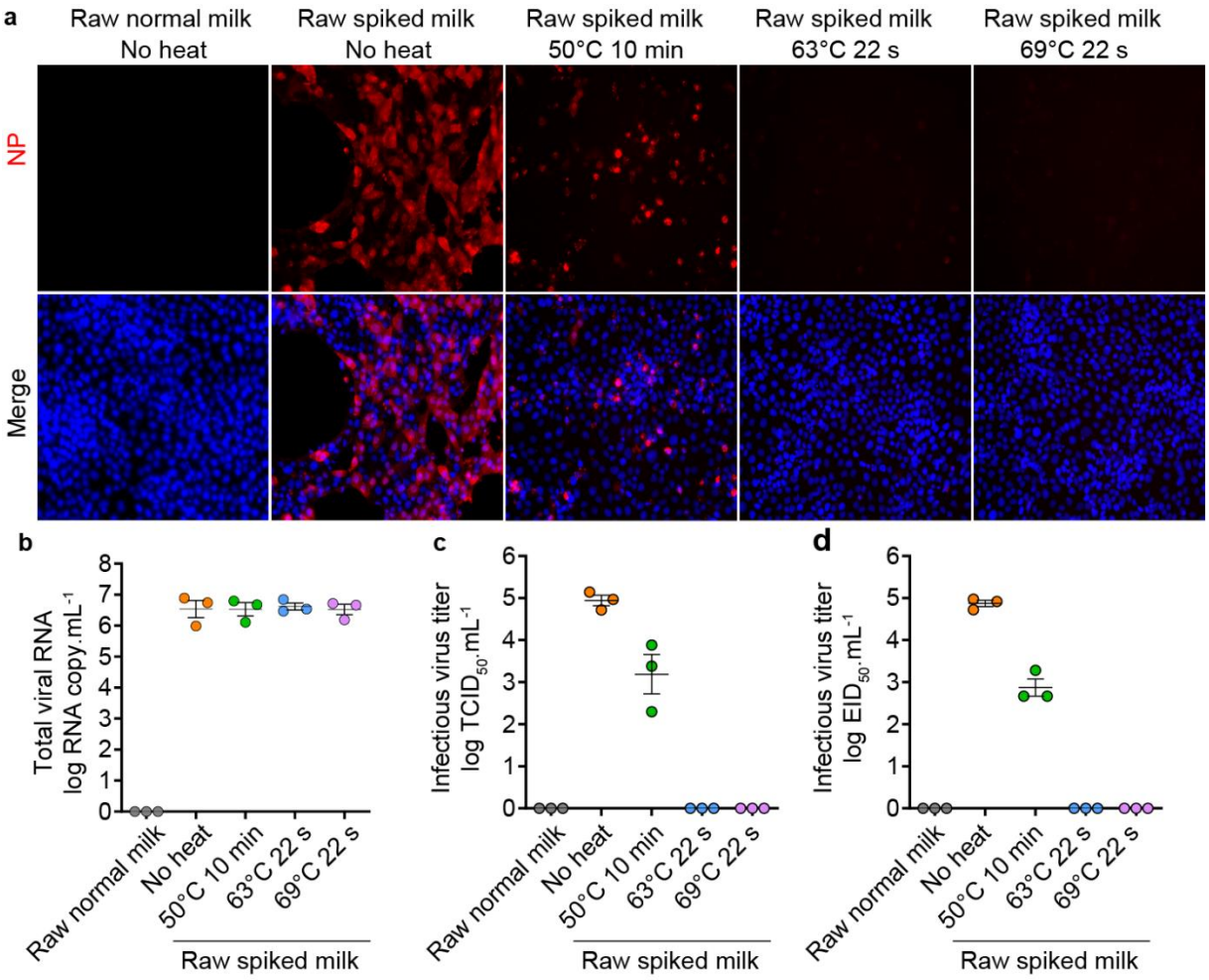


Fig. 3. Evaluation of thermization conditions on inactivation of HPAI H5N1 virus in raw milk. Thermal inactivation of HPAI H5N1 virus in raw normal milk spiked (1:20 ratio) with HPAI H5N1 using a thermocycler. **a.** IFA images of Cal-1 cells inoculated with control or heat-treated HPAI-spiked raw normal 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).

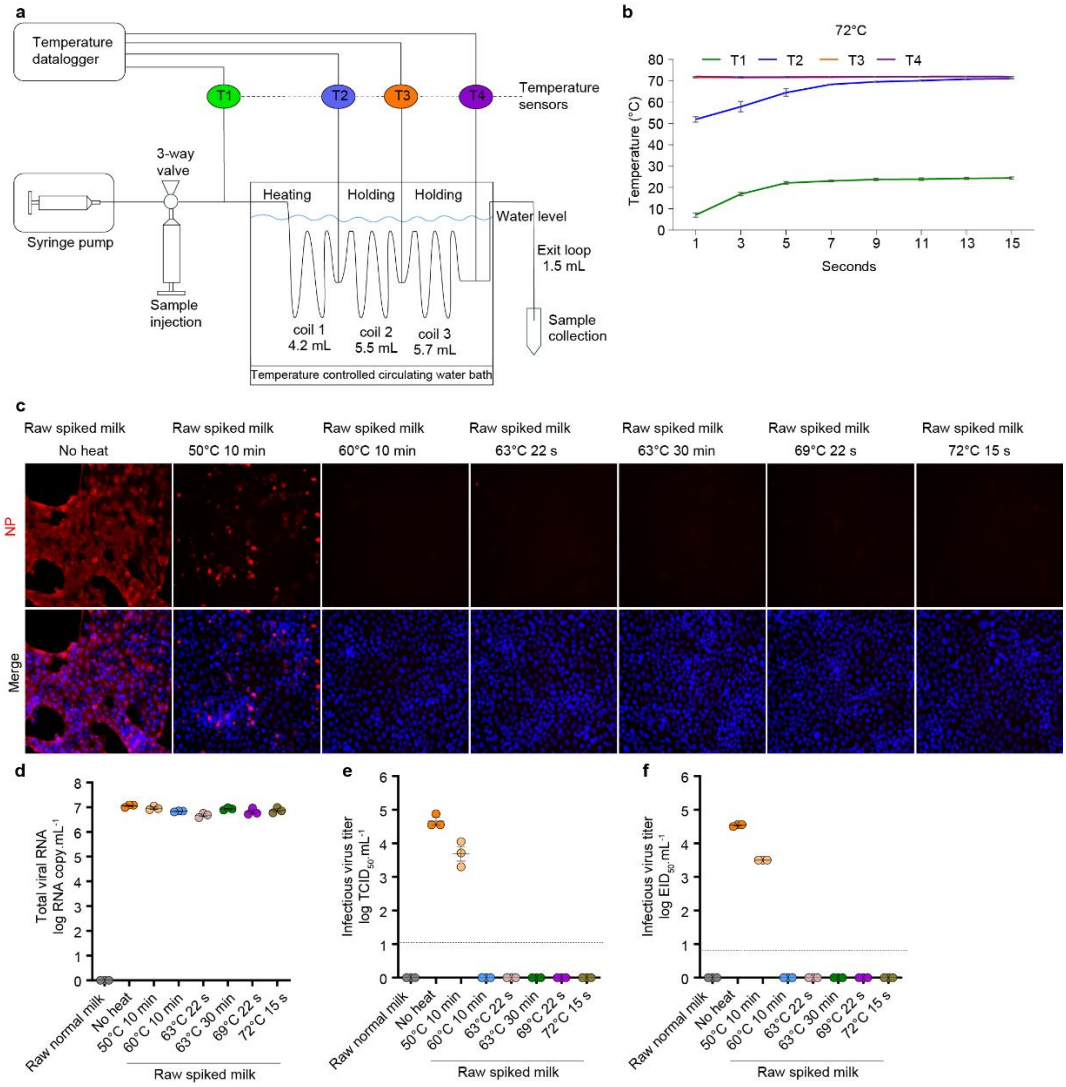


Fig. 4. Thermal inactivation of HPAI H5N1 in virus-spiked milk samples using a submerged coil heating system. **a**. Schematic representation of the submerged coil heating system. **b.** Representative plot showing temperature changes over time in milk samples injected in the submerged coil system during 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 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).

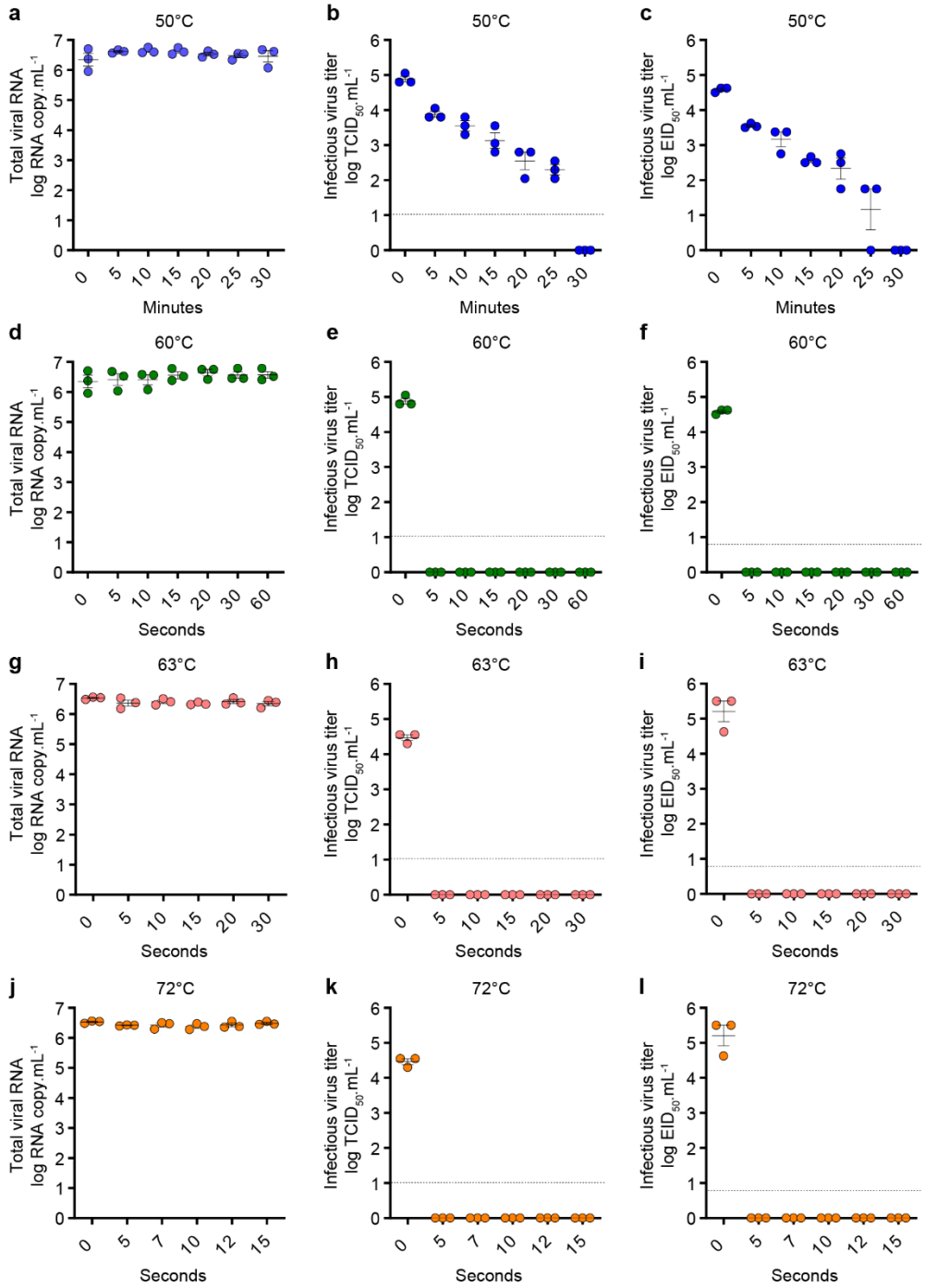


Fig. 5. Kinetics of HPAI H5N1 virus inactivation in milk using submerged coil heating system. Raw normal milk was spiked with HPAI H5N1 at 1:20 ratio and heat treated at 50°C (**a-c**), 60°C (**d-f**), 63°C (**g-i**) and 72°C (**j-l**) with a submerged coil heating system. Sequential samples were collected 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 cells ($TCID_{50}.mL^{-1}$) and embryonated chicken eggs ($EID_{50}.mL^{-1}$). Data represents observations (dots) from three independent experiments (n=3) overlayed with the mean (horizontal line) and ± SEM (whiskers).

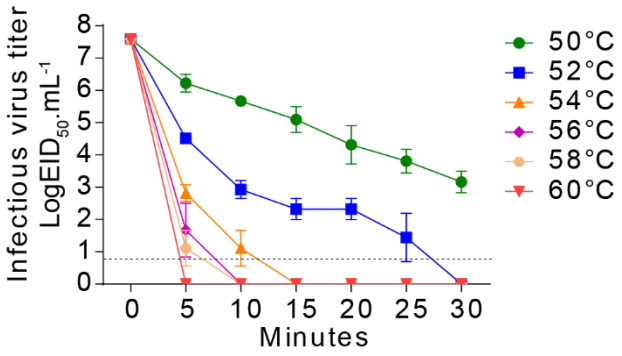
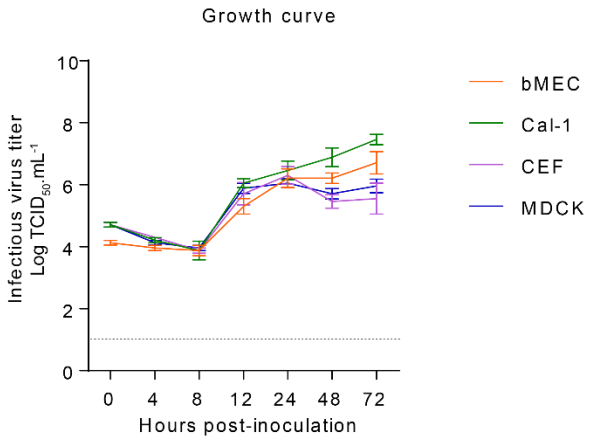
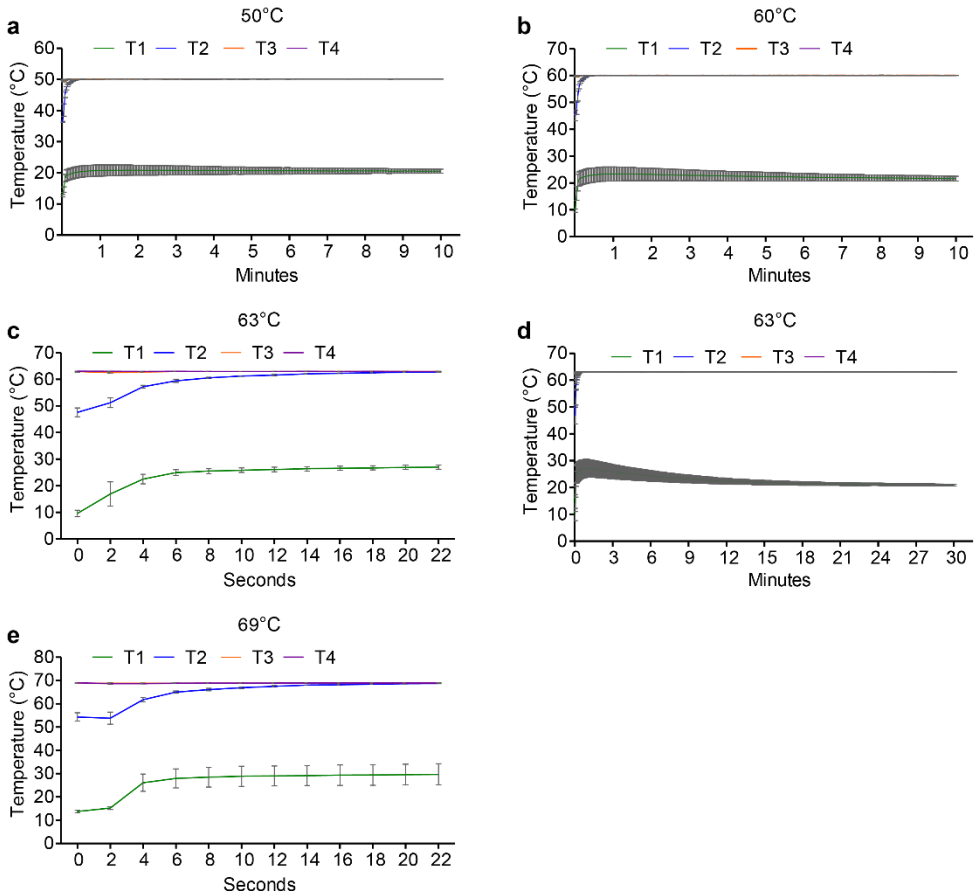


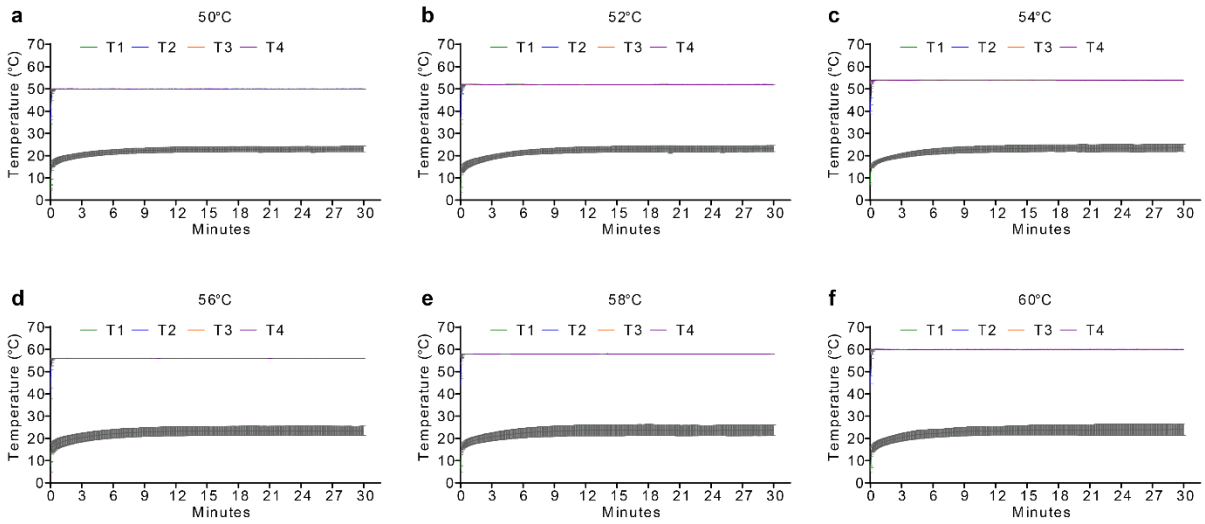
Fig. 6. Thermal resistant constant (*z*-value) of HPAI H5N1 virus in raw milk. Raw normal milk was spiked with HPAI H5N1 at 1:20 ratio and heat treated at 50°C, 52°C, 54°C, 56°C, 58°C and 60°C with a submerged coil heating system. Sequential samples were collected at the indicated time points and subjected to virus titration using embryonated chicken eggs ($EID_{50}.mL^{-1}$). The EID_{50} titers were used to calculate the thermal resistant constant *z*-value of HPAI H5N1 in raw milk. Data represents observations (dots) from three independent experiments (n=3) overlayed with the mean (horizontal line) and ± SEM (whiskers).



Extended Data Fig. 1. Multicycle growth curve of HPAI H5N1 TX2/24. Primary bovine mammary epithelial cells (bMEC), bovine uterine epithelial cells (Cal-1), primary chicken embryo fibroblast (CEF) and Madin-Darby canine kidney (MDCK) cells were infected (MOI 0.1) with HPAI H5N1 TX2/24 and virus titers were determined at indicated time points by limiting dilution method and expressed as $TCID_{50}.mL^{-1}$. Data indicates mean ± SEM, n = 3, three independent experiments.



Extended Data Fig. 2. 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.



Extended Data Fig 3. Line graph showing temperature changes over time in injected milk samples during heat treatment in the submerged coil heating apparatus at 50°C (**a**), 52°C (**b**), 54°C (**c**), 56°C (**d**) 58°C (**e**) and 69°C (**f**) over 30 min. Data are presented as mean ± SEM of 3 independent experiments.