# Evaluation of High-Pressure Processing in Inactivation of the Hepatitis E Virus

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## Abstract

Hepatitis E virus (HEV) causes acute hepatitis with approximately 20 million cases per year globally. While HEV is endemic in certain regions of Asia, Africa and South America, it is considered an emerging foodborne pathogen in developed countries. Based on genetic diversity, HEV is classified into different genotypes, with genotype 3 (HEV-3) being most prevalent in Europe and North America. The transmission of HEV-3 has been shown to be zoonotic and mainly associated with the consumption of raw or undercooked pork products. Herein, we investigated the efficacy of high-pressure processing (HPP) in the inactivation of HEV-3 using a cell culture system. HPP has been indicated as a promising nonthermal pathogen inactivation strategy for treatment of certain high-risk food commodities, without any noticeable changes in their nature. For this purpose, we treated HEV-3 in media as well as in artificially inoculated pork pâté, with different conditions of HPP: 400 MPa for 1 and 5 minutes, as well as 600 MPa for 1 and 5 minutes, at ambient temperature. In general, we observed approximately a 2-log reduction in HEV load by HPP treatments in media; however, similar treatment in the pork pâté resulted in a much lower reduction in viral load. Therefore, the efficacy of HPP treatment in the inactivation of HEV-3 is matrix-dependent.

**Importance:** HEV is an emerging foodborne pathogen in industrialized countries, and its transmission is associated with the consumption of contaminated undercooked pork product. In this work, we employed an infectivity assay to investigate the potential of high-pressure in inactivation of HEV in media and ready-to-eat pork pâté. We demonstrated that the effect of HPP on inactivation of HEV depends on the surrounding matrix.

**Keywords**: Hepatitis E virus, high-pressure processing, infectivity assay, droplet-digital RT-PCR

## Introduction

HEV is a single-stranded RNA virus with positive polarity belonging to the *Hepeviridae* family (1). The HEV genome has 3 open reading frames (ORFs): ORF1 encodes a long non-structural polyprotein with multiple functions; ORF2 encodes the viral capsid protein; and ORF3 encodes a small phosphoprotein with structural and non-structural functions (2), (3). The *Hepeviridae* contain two genera *Orthohepevirus* and *Piscihepevirus,* which infect a wide range of vertebrate hosts (4). Four genotypes (HEV-1, HEV-2, HEV-3 and HEV-4) of the species *Orthohepevirus* A are associated with human illness. HEV-1 and HEV-2 are restricted to humans and are prevalent in regions with poor water sanitation, such as the developing countries of Asia, Africa, South and Central America (5, 6). On the other hand, HEV-3 and HEV-4 are considered to be zoonotic pathogens as they have a much wider range of mammalian hosts including, among others, domestic and wild swine and ruminants (7-9). Hepatocytes have been identified as the primary sites of HEV replication, but the virus can replicate in other tissues such as epithelial cells of the small intestine, placenta, and muscle (10-13).

Clinical manifestation of HEV can vary depending on virus genotype and the host. It is generally believed that the majority of HEV infections are subclinical (14). In symptomatic cases, HEV most commonly presents as a self-limiting, acute infection (6, 15). However, chronic HEV infection can occur after infection with HEV-3, and possibly HEV-4, specifically in immunosuppressed patients, such as human immunodeficiency virus patients or those receiving immunosuppressing treatment (16-18). In recent years, the incidence rate of HEV-3 infection has increased in industrialized countries, likely through zoonotic exposure (19). Due to the lack of surveillance data, the actual HEV incidences and fatalities per country are often unknown, and therefore the true burden of HEV disease remains unclear (19, 20).

59   Multiple lines of evidence indicate that infection with HEV-3 is common among domestic swine

60   in developed countries (Reviewed in (21)), however HEV-3 viremia in swine does not cause any

61   noticeable clinical symptoms (22-24). HEV-3 infection of domestic swine can potentially result

62   in contamination of pork products. The reported prevalence of contaminated pork products varies

63   from less than 1% to more than 50%, depending on the region and the tested commodity

64   (reviewed in (21)). In a previous study conducted by, our laboratory it was observed that 10.5%

65   of sampled raw pork livers, and 47% of the sampled commercial pâté, marketed in Canada, were

66   positive for HEV RNA (25). Because of this high prevalence, efficient strategies to inactivate

67   HEV in ready-to-eat pork products should be considered in order to prevent foodborne HEV

68   infection.

69   High pressure processing (HPP) is a "nonthermal pasteurization" technique, which can inactivate

70   foodborne pathogens within certain commodities such as ready-to-eat meats and fruit juices to

71   increase their shelf life or improve safety (26) . It is generally believed that high-pressure

72   treatment denatures the viral capsid proteins and therefore incapacitates the viral particles from

73   attachment and penetration to the host cells (26, 27). However, due to the lack of reliable

74   infectivity assays, most HEV inactivation studies to date have been limited to using surrogate

75   viruses (27, 28). Recently, successful replication of HEV-3c strain 47832c (GenBank accession #

76   KC618403), in A549/D3 cells was demonstrated by Johne and coworkers (29, 30). This system

77   has been employed to study the temperature sensitivity of HEV (30), demonstrating a potential

78   for this system to be used in other HEV inactivation studies. Herein, we describe the

79   employment of this HEV infectivity assay to examine the effect of HPP treatment on HEV

80   infectivity in both cell culture media and ready-to-eat pork pâté.

## Materials and Methods

### Cells & Viruses

A549/D3 human lung carcinoma cells, kindly provided by Dr. R. Johne (German Federal Institute for Risk Assessment, Berlin), were cultured in Minimum Essential Media (MEM) (Gibco, MA, USA), supplemented with 1% non-essential amino acids, 1% glutamine, 0.5% gentamicin, and 10% fetal bovine serum (FBS) (Gibco, MA, USA).

The optimal cell density of A549/D3 cells per well was determined to be $4\times10^4$ cells per well of a 96-well plate. The plate was then incubated for 2 days at 37˚C and 5% CO2. Growth media was replaced with fresh media and cells were incubated under the same conditions for another 3 days, until the infectivity assay was performed.

### Sample preparation for HPP treatment

Sterile polyethylene tubes (Tygon ®) 1.5 cm in length were filled with 200 µl of cell growth media containing $2\times10^6$ genome copies of HEV-3 strain 47832c and heat sealed. Triplicate tubes were prepared in sets for each treatment duration (0, 1, or 5 min), for a total of 9 tubes. The tubes for each time point were then placed in polyethylene (PE) bags containing 10% bleach, to inactivate viral particles in the event of leak or rupture from the primary container. The sample bags were then heat-sealed, while minimising air bubbles in the bleach solution. Prepared sample bags were stored on ice until the HPP treatment.

Pork pâté samples were prepared from commercial product obtained from a local grocery store. Individual samples of 2 g were weighed out to prepare triplicate samples. An uninoculated pâté sample was retained as a negative control. Samples were inoculated with 250 µl of cell culture medium containing approximately $4\times10^7$ genome copies, which was spread over the entire

5

103    surface area of the sample. . Inoculated samples were dried for 10 min in a biosafety cabinet,

104    prior to being placed in individual PE bags, which were heat-sealed with minimal air space.

105    Triplicate samples for each treatment duration (0 min, 1 min, 5 min) were then placed in a

106    second PE bag containing 10% bleach, and stored on ice prior to HPP treatment.

107    **HPP Treatment**

108    High-pressure processing was implemented using a high-pressure pilot unit manufactured by

109    Dustec Hochdrucktechnik GmbH (Wismar, Germany), with a 1-liter pressure vessel and water as

110    the pressure medium. The rate of pressurisation was 10 MPa/s and rate of depressurisation was -

111    20 MPa/s. Sample packages were pressurized to 400 MPa or 600 MPa with a hold time at

112    maximum pressure of 1 or 5 min. As determined by three thermocouples inside the pressure

113    vessel, the temperature of the pressure medium was initially 24.0 °C (standard deviation (SD) 0.3

114    °C, n=4). Adiabatic heating during pressurisation resulted in an average temperate increase of

115    8.2 °C (SD 0.1 °C, n=2) when pressurised to 400 MPa and 12.9 °C (SD 0.1 °C, n=2) when

116    pressurised to 600 MPa.

117    **Virus Extraction**

118    The ISO-15216-1:2017 (31) method was used to extract HEV from pork pâté samples post HPP

119    treatment. Briefly, the pâté samples were transferred to stomacher bags with a filter compartment

120    and 16 mL of Tris Glycine Beef Extract (TGBE) was added respectively. The stomacher bags

121    were then incubated on a rocking plate at room temperature for 20 min. The resulting suspension

122    was centrifuged at 10,000 × g for 30 min at 4 ˚C. The supernatant pH was balanced using

123    approximately 110 μl of 12 N HCl. 5 × polyethylene glycol 6000 (PEG)/NaCl of ¼ volumes of

124    the weight of each sample was added to each tube and the samples were incubated on ice on a

125    rocking plate for 1 hour. Post incubation, samples were centrifuged at 10,000 × g for 30 min at 4

6

126 °C and the supernatant was discarded. The pellets containing the virus particles were suspended

127 in 500 μl PBS and stored at -80 °C until required for the infectivity assay.

**Determining the Limit of Quantification**

129 In order to determine the limit of quantification of the infectivity assay, cell culture-adapted

130 HEV-3 strain 47832c at ten concentrations from $5 \times 10^2$ to $1 \times 10^6$ genome copies per well were

131 used to infect A549/D3 cells in triplicate experiments (29, 30). The infected and control cells,

132 which were not exposed to viral particles, were cultured for 14 days. The media supernatant was

133 then collected and the HEV RNA levels were analysed by droplet-digital RT-PCR (ddRT-PCR).

**RNA Isolation and Quantification**

135 The Viral RNA Mini Kit (Qiagen, Mississauga, Ontario, Canada) was used to extract RNA from

136 the collected infectivity assay growth media. Quantification of recovered RNA was conducted as

137 previously described using Bio-Rad droplet digital PCR (ddRT-PCR) technology (25, 32).

**DNA sequencing**

139 Conventional RT-PCR was carried out using the HEV-11 primers (33),  which amplifies the

140 region between the positions 5468-6018 of the HEV-3 strain 47832c. Gel-purified RT-PCR

141 products were sequenced directly using the BigDye® terminator v 3.1 DNA sequencing kit

142 (Applied Biosystems, Foster City, California) according to manufacturer's instructions.

143 Fluorophore-labelled reactions were purified using the Wizard®MagneSil® Sequencing Reaction

144 Clean-up System (Promega, Madison, Wisconsin). Samples were sequenced in both directions

145 using a 3130xl Genetic Analyzer (ThermoFisher Scientific). HEV-positive sequences were

146 determined by querying NCBI BLAST and edited using BioEdit (Ibis Biosciences, Carlsbad,

147 California).

148    Multiple sequence alignments were performed using both the Multiple Sequence Comparison by

149    Log-Expectation (MUSCLE) (34) and Clustal W (35) included in the MEGA6 software (36) .

150    The sequences obtained in this study have been deposited in GenBank under Accession Numbers

151    **Data analysis**

152    All experiments were performed in triplicate. Statistical analysis was performed by Microsoft

153    Excel 2016. Paired student's *t*-test was conducted to obtain *P* values.

154    # Results

155    **Limit of Quantification**

156    The correlation between the inoculated HEV genome copy number and the harvested genome

157    copy number at 14 days post infection (d.p.i) is shown in Figure 1. The relationship between the

158    two is linear over the range studied with a $r^2$ value of 0.9823, this demonstrates that the amount

159    of harvested HEV RNA is directly correlated to the of HEV inoculum. The limit of

160    quantification by this method was determined to be $1 \times 10^4$ genome copies per well (100 gc/µl) of

161    the inoculated virus, and inoculation with titres below this amount did not reliably and

162    reproducibly produce quantifiable progeny virus at 14 d.p.i. Importantly, these data suggest that

163    on average, 1 in $10.2 \pm 4.8$ of the inoculated genomes is capable of replication in cell culture. In

164    other words, the infectivity ratio of the virus is 1 in $10.2 \pm 4.8$ genome.

165    **HEV inactivation in cell culture media**

166    In commercial food processing, HPP is applied to meat products with pressures typically ranging

167    between 400 and 600 MPa for 1 to 10 min (37). To determine the role of pressure and hold time

168    on the inactivation of HEV by HPP, HEV-3 strain 47832c, in cell culture media, were treated at

169    pressure levels of 400 MPa and 600 MPa for 1min and 5 min starting at 24 °C. The undiluted

170    and 1:10 diluted HPP-treated viral solutions, along with untreated controls, were used to infect

171    A549/D3 cells in duplicate as described above. The decrease in infectious HEV particles was

172    determined by comparing the reduction in HEV RNA at 14 d.p.i in HPP-treated samples with the

173    untreated controls. As shown in Figure 2, reductions of 1.6±0.33 log and 1.93±0.29 log in

174    infectious viral particles were observed for the samples that were treated at 400 MPa for 1 min

175    and 5 min, respectively. Increasing the pressure to 600 MPa resulted in a slight but not

176    statistically significant increase in viral inactivation; 2.27±0.03 log and 2.2±0.28 log reduction

177    for 1 min and 5 min treatments, respectively (Figure 2). Neither varying the treatment pressure

178    (400 MPa or 600 MPa) nor the hold time at maximum pressure (1 min or 5 min) resulted in

179    statistically significant reductions in the viral inactivation ($P > 0.1$).

**Examining amino acid variation in the capsid protein**

181    The HEV capsid protein consists of 660 residues and 4 main structural and functional domains;

182    N, S, M and P (38, 39). To examine whether the capsid protein of the viruses that survived the

183    HPP treatment is different from that of the input or the untreated viruses, we compared the amino

184    acid sequence of the partial capsid protein encompassing the N and S domains of the viruses

185    treated with 600 MPa for 1 min hold time and 600 MPa for 5 min hold time with the input and

186    untreated viruses. As shown in Figure 4, no synonymous change was observed between the

187    treated and untreated viruses within the sequenced range.

**HEV inactivation in ready-to-eat pork pâté**

189    In order to investigate whether food matrices can protect from or potentiate the inactivation of

190    HEV by HPP, experiments were conducted with a high-risk ready-to-eat pork product,

191    artificially inoculated pork pâté. HPP treatment at 400 MPa or 600 MPa for up to 5 min did not

192    cause any noticeable change in the appearance of pâté samples (Supplementary Figure 1). HEV

193     was extracted using the ISO-15216-1 method and was used to infect A549/D3 cells. At 14 d.p.i

194     the media was harvested and examined for the presence of viral RNA using dd RT-PCR. The

195     effect of the HPP treatment was determined by comparing the viral load in treated samples

196     against the untreated samples. Surprisingly, HPP treatment of pork pâté at 400 and 600 MPa for

197     1 min and 5 min, resulted in significantly lower reductions in viable HEV than observed in cell

198     culture media. At 400 MPa the reduction in viable HEV was only $0.48\pm0.14$ log and $0.46\pm0.13$

199     log, , and at 600 MPa $0.39\pm0.08$ log and $0.52\pm0.24$ log, respectively for 1 min and 5 min

200     treatments (Figure 3). As observed in culture media, no significant difference in virus

201     inactivation was observed between 1 and 5 min treatment at the same pressure ($P > 0.1$), also

202     increasing pressure to 600 from 400 MPa did not result in increased HEV inactivation in pork

203     pâté ($P > 0.1$).

## Discussion

205     Herein, we employed an infectivity-based model for examining the infectious dose of HEV-3

206     strain 47832c in cell culture. Using this system, we demonstrated that approximately 1 in $10 \pm 5$

207     viral genomes is capable of replicating in A549 cells. This finding is in line with the high

208     infectivity of other foodborne viruses such as norovirus and hepatitis A virus (40-42).

209     We next employed this system to investigate HEV inactivation by HPP treatment. The untreated

210     and treated virus stocks were used to infect the A549 cells and the infected cells were examined

211     for the production of progeny virus in the culture. Using this system, we demonstrated that an

212     approximately 2-log reduction in viral load can be accomplished by treatment of HEV in media

213     at 400 MPa with a 1 min hold time. Increasing the pressure to 600 MPa or the hold time to 5 min

214     did not have any significant effect on the reduction of viral load. However, HEV in artificially

215    contaminated pâté was protected from HPP treatment, as the reduction in infectious HEV

216    particles was less than 0.5 log.

217    To the best of our knowledge, this is the first published study to quantify the inactivation of HEV

218    following HPP treatment. HEV response to 500 MPa for 15 min has been previously investigated

219    with RT-qPCR viability markers (PMAxx and platinum chloride, PtCl4-RT-qPCR), but was only

220    able to report the presence of intact viral particles post treatment (43). The inactivation by HPP

221    of other foodborne viruses, including norovirus, Hepatitis A virus (HAV), and surrogates for

222    foodborne viruses has been investigated (26, 44-46). The sensitivity of specific viruses to high-

223    pressure treatment can vary significantly. Kingsley et al. reported that HAV in cell culture media

224    under a 5 min hold period was stable at pressures up to 300 MPa, but inactivation increased with

225    increasing pressure between 300 and 450 MPa, to maximum of 6 log reduction. In contrast, in

226    feline calicivirus (FCV) (a surrogate for norovirus) 3 log reductions were observed at 200 MPa

227    and no infectious particles were recovered following treatment at 275 MPa (44). Inactivation of

228    norovirus suspended in buffer has been reported at pressures in excess of 200 MPa (5 min hold, 4

229    °C), but the sensitivity to pressure was variable between the four strains studied, with the most

230    sensitive strain reduced by 4 log at 600 MPa and the least sensitive by only 1 log under the same

231    conditions (45) .

232    HEV is a quasi-enveloped virus (47, 48), and the presence of a lipid envelope may have a

233    protective role. If so the impact HPP could potentially be enhanced in the presence of detergents

234    or other membrane disrupting molecules. Alternatively, differences in pressure resistance

235    between strains of the same virus may be related to amino acid variability in capsid proteins, or

236    lipids composing membranes.

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237    The sequence analysis of partial capsid protein revealed that there is no amino acid change

238    between the treated and untreated viruses within the N and S domain. However, to determine

239    whether the capsid of the surviving viruses are different from the untreated viruses, full capsid

240    sequence analysis is required. In this study, our attempts to retrieve the full capsid sequence from

241    the treated samples were not successful. Nevertheless, we cannot rule out the possibility of

242    reversion of mutations during the culture period (14 d).

243    In this study, we observed that HEV in pork pâté was protected from HPP treatment, compared

244    to HEV in cell culture media. The dependency on the surrounding matrix of the response of

245    bacterial cells to HPP treatment is a well-established phenomenon, with salt concentration, pH,

246    fat content, and the presence of specific molecules reported to affect cell survival the variables

247    (49). Similar observations have been made for viruses, with pH, temperature and solute

248    concentration reported as variables in the response of norovirus to HPP (45). The presence of

249    food components has been demonstrated to protect viral capsids from HPP denaturation (50). A

250    further demonstration of the challenge in extrapolating studies in model systems to compel

251    foods, a human volunteer study with oysters inoculated with 4 log PFU of norovirus (GI.1.

252    Norwalk) found that a treatment of 400 MPa (5 min hold, 6 °C) was insufficient to protect

253    volunteers, though 600 MPa was protective for all volunteers (51).

254    The protective effect of food components against viral capsid denaturation has already been

255    demonstrated (50). It has also been reported that fat increases the stability of hepatitis A virus in

256    skim milk and cream against heat treatment (52); however, whether the fat and salt content of the

257    treated matrix affects the structural integrity of HEV and its sensitivity towards pressure, needs

258    to be further investigated.

259  Our data demonstrated that the viral titre post HPP treatment of pâté under 600MPa for 5 min

260  reaches to $1.5 \times 10^3$ in cell culture. This indicates that replication of virus occurred, and therefore

261  the elimination of HEV infectivity was not complete. In another study, it was shown that the

262  treatment of HEV solution at 500 MPa for 15 min did not result in complete inactivation assayed

263  by using viability markers (PMAxx and platinum chloride, PtCl4-RT-qPCR) (43). Knowing that

264  the virus is capable of replication (and therefore has the potential to cause illness) is important

265  for interpretation of surveillance and inactivation studies on HEV to inform risk assessment and

266  mitigation (28) . Especially that the dose-response relationship of HEV is unknown, and it is not

267  clear which level of infectivity reduction is required to prevent infection in human. Therefore,

268  the generation of more detailed data on the infectivity reduction for different HEV strain-matrix

269  combinations would enhance our understanding of HEV stability in the environment and in

270  foods. (29).

271  In summary, we have demonstrated that 1) there is a direct and linear correlation between the

272  viral titres used to infect A549/D3 cells and the harvested virus at 14 d.p.i 2) the effect of HPP

273  on inactivation of HEV depends on the surrounding matrix.

274  **Acknowledgements**

279

280    **Figure Legends**

281    **Figure 1.** Correlation between the inoculated HEV-3c strain 47832c  (genome copy number) and

282    the harvested HEV (genome copy number) 14 d.p.i in A549/D3 cells.

283    **Figure 2.** The effect of HPP treatment on HEV-3c strain 47832c in cell culture media. The

284    samples containing $2 \times 10^6$ genome copies were treated at 400 MPa and 600 MPa for 1 and 5 min

285    at ambient temperature. The effect is shown in comparison to the untreated viral stock.

286    **Figure 3.** The effect of HPP treatment on HEV-3c strain 47832c in ready-to-eat pork pâté. The

287    samples containing $4 \times 10^7$ genome copies of HEV were treated at 400 MPa and 600 MPa for 1

288    and 5 min at ambient temperature. The effect is shown in comparison to the untreated but

289    inoculated samples.

290    **Figure 4**. Amino acid sequence alignment of the N domain of the capsid protein (ORF2). The

291    input, the untreated, treatment at 600 MPa for 1 min hold time and 600 MPa for 5 min hold time.

292    **Supplementary Figure 1.** Visual assessment of HPP processed pork pâté samples. Samples

293    were treated in triplicate at 400 MPa (A & B) and 600 MPa (C & D) for 1 min and 5 min

294    respectively at ambient temperature. Visual inspection and documentation were conducted post-

295    treatment to detect any changes in food quality or appearance (size, colour, texture, and secretion
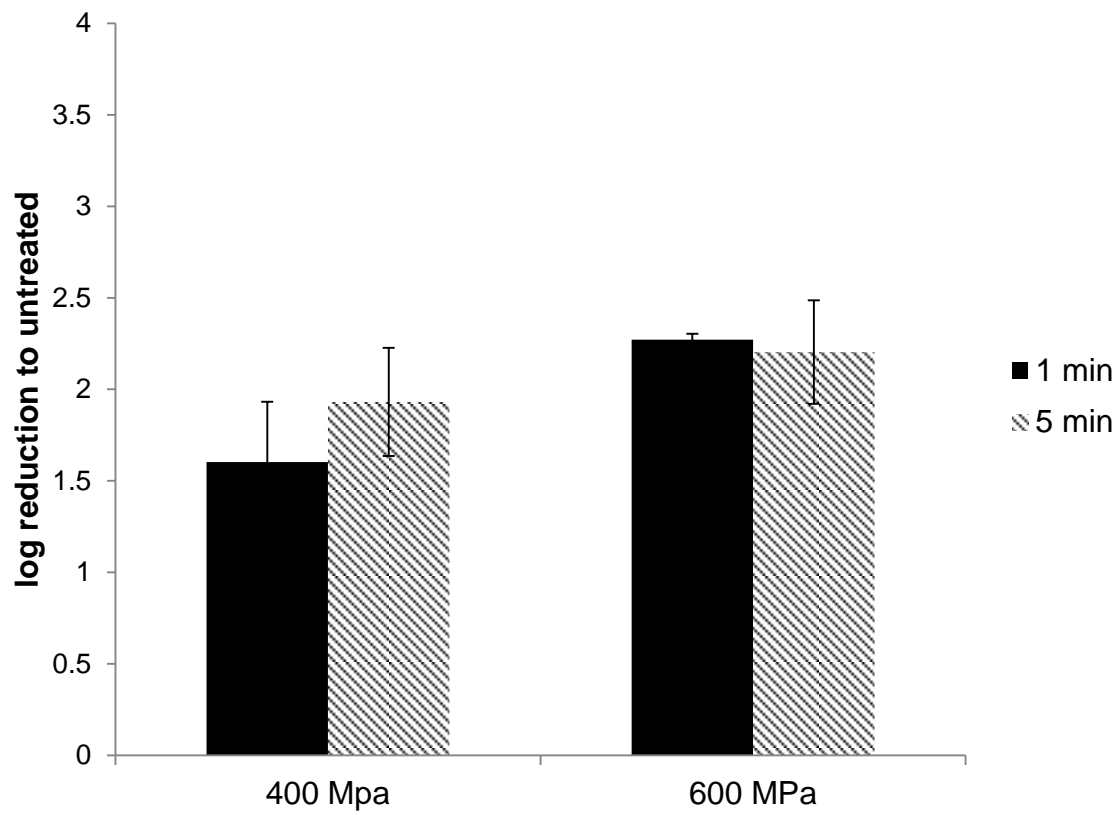
296    of fluids**).**

297    **Figure 1**
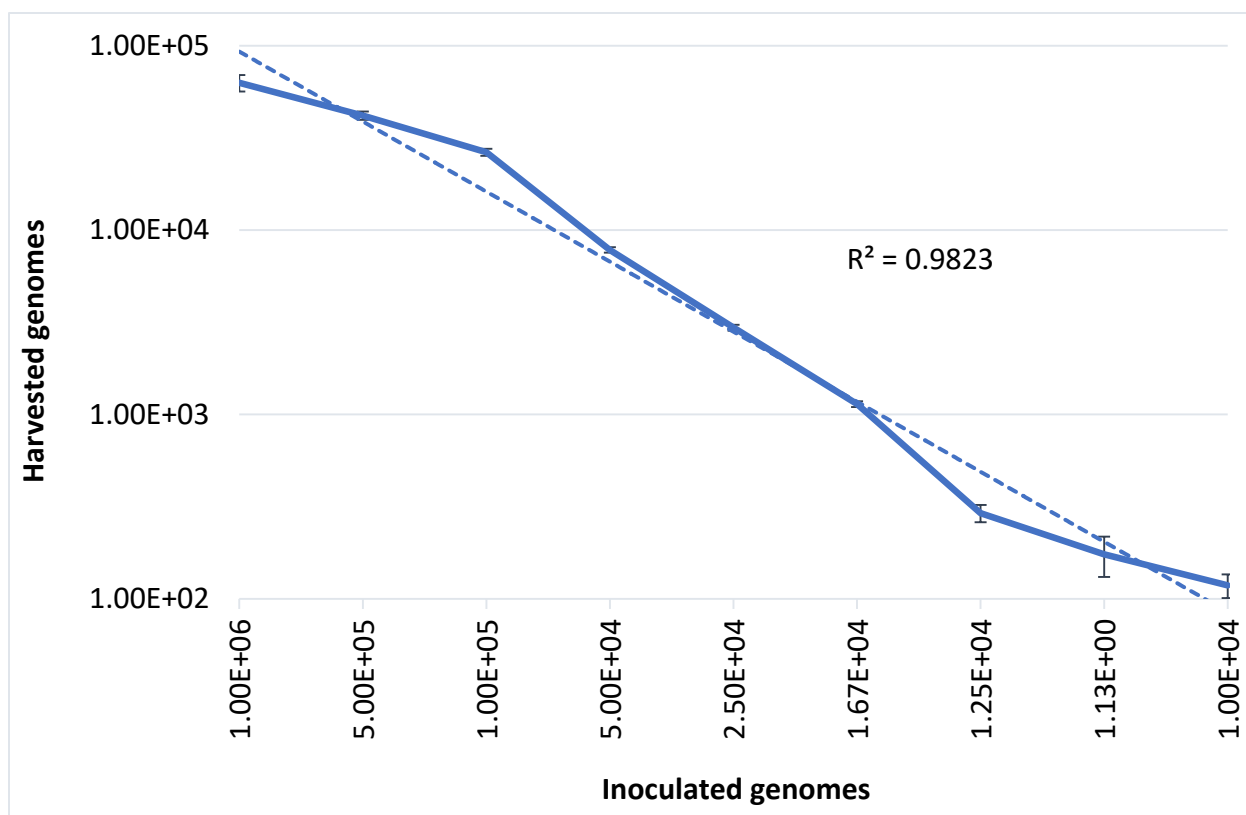


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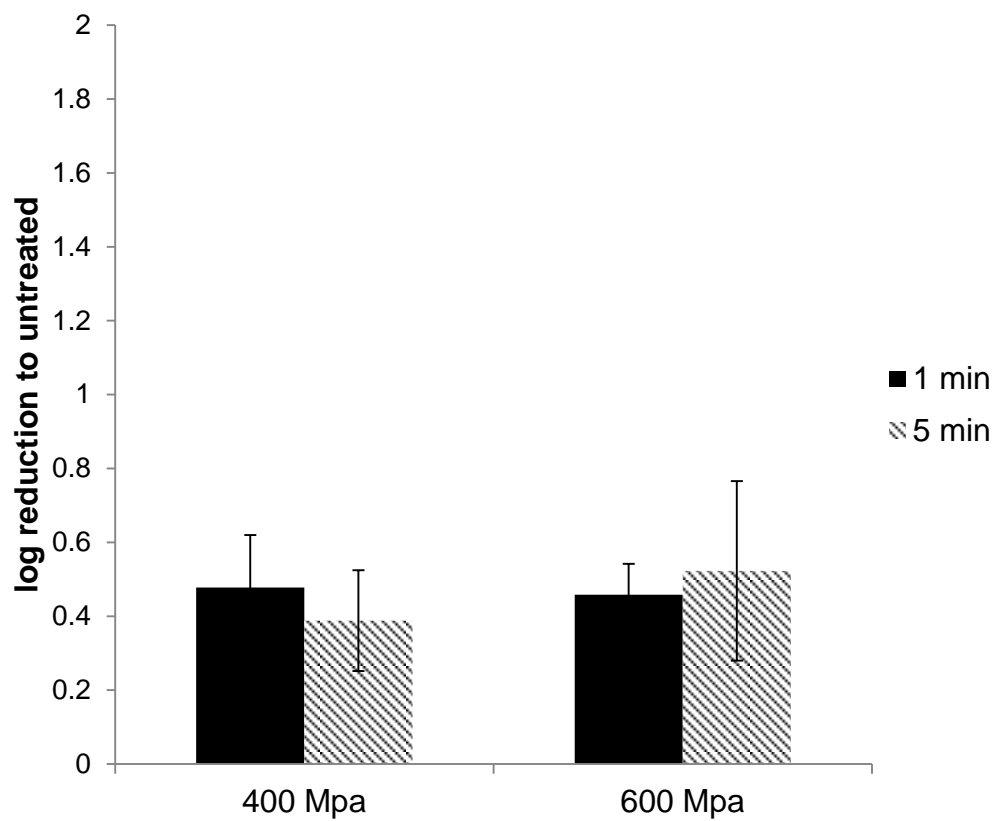
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302    **Figure 2**
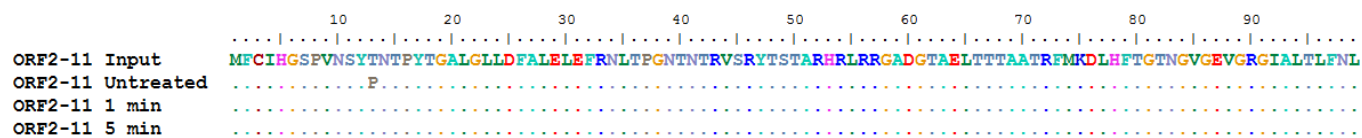


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307     **Figure 3**



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311   **Figure 4**

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                            10        20        30        40        50        60        70        80        90
                     ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....
     ORF2-11 Input   MFCIHGSPVNSYTNTPYTGALGLLDFALELEFRNLTPGNTNTRVSRYTSTARHRLRRGADGTAELTTTAATRFMKDLHFTGTNGVGEVGRGIALTLFNL
     ORF2-11 Untreated ...........P.......................................................................................
     ORF2-11 1 min   ..................................................................................................
     ORF2-11 5 min   ..................................................................................................
```
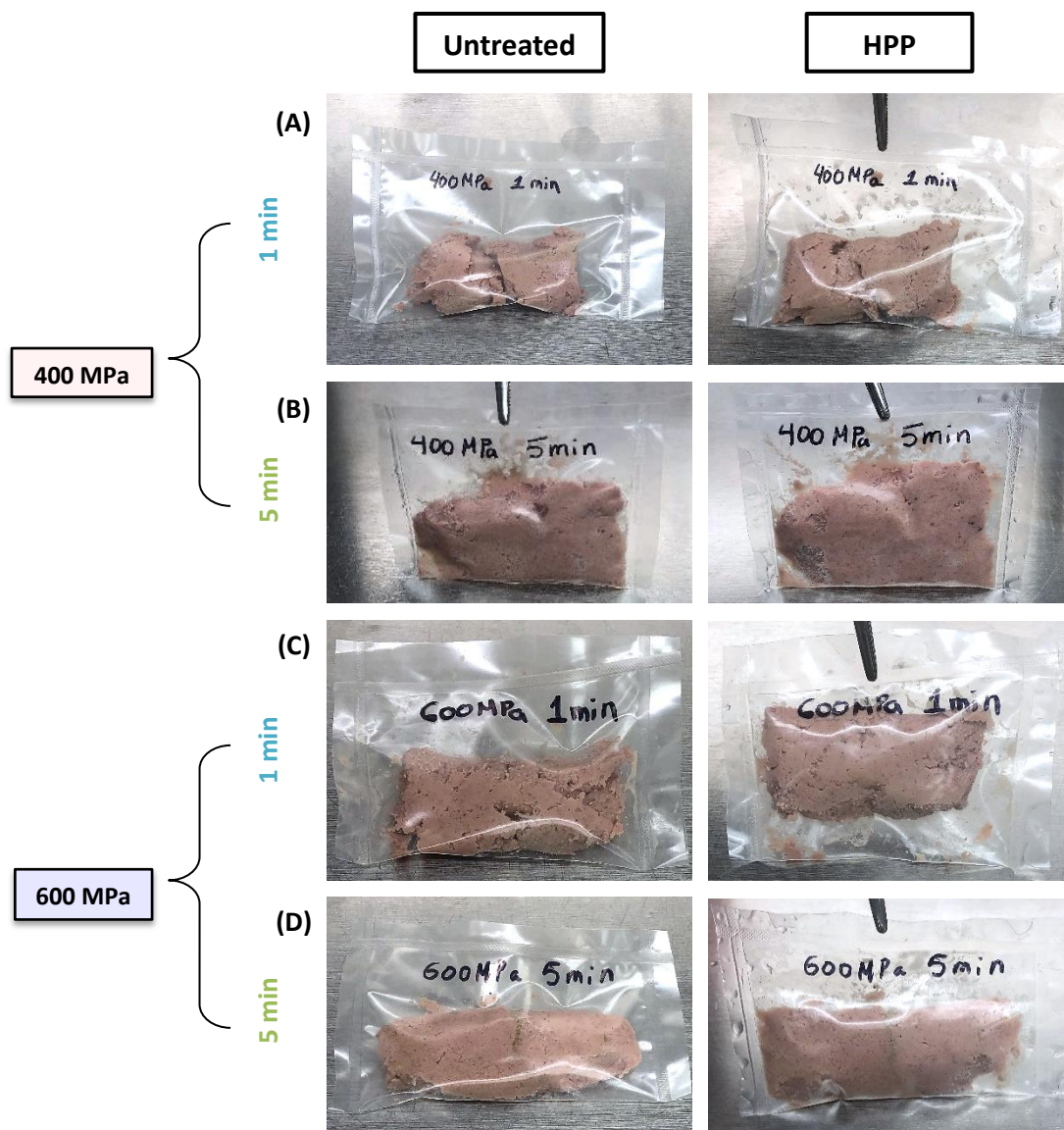
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315 **Supplementary Figure 1**

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