Court File No.: **M40828**

COURT OF APPEAL FOR ONTARO

B E T W E E N:

HER MAJESTY THE QUEEN

(Respondent)

- and –

MICHAEL SCHMIDT

(Applicant)

NOTICE OF MOTION

TAKE NOTICE that the Applicant will make a motion before the judge hearing the motion for leave to appeal at Osgoode Hall, 130 Queen Street West, Toronto, Ontario on Thursday, July 26, 2012 or as soon thereafter as this motion can be heard.

PROPOSED METHOD OF HEARING: The motion is to be heard orally. The estimated total length of time for oral argument is 15 minutes, to be incorporated as part of the 3 hours already allocated to the hearing of the leave motion.

THE MOTION IS FOR:

Leave to re-open the cross-examination of the Respondent's expert witnesses Dr. Mansell William Griffiths and Dr. Jeffrey Boyd Wilson by way of oral, out-of-court examination under oath, in order to question them on a newly published scientific study that was unavailable at the time of trial and that contradicts the evidence each witness gave pertaining to the pathogen e.coli 0157:H7 and pasteurization; and leave to introduce the transcripts of the cross-examinations as fresh evidence on the appeal.

THE GROUNDS FOR THE MOTION ARE:

1. The oral evidence of Dr. Mansell William Griffiths given at trial on January 30, 2009 pertaining to the pathogen E.coli 0157:H7 (occasionally found in raw milk) was as follows:

> "I think it's been well demonstrated in the literature that you can get new types of—of organisms that—that were—that—that were non-pathogen before it can take on new characteristics. And a good illustration is—is the organism that we've talked a lot about E-coli 157-8-7 (sic) and Doctor Beals explained what the 0157-8-7 (sic) designation was. That organism has become more of a concern because it has acquired genes for the production of toxin from a closely related organism called "Shigella" and it's been—it's done that through the—the transfer of genetic—through the transfer of genetic material....[the pasteurization process] It kills 0157... E.coli 157 is killed.by pasteurization, yes."

- Motion Record, Volume II, Tab 19, pages 20-22

2. The affidavit evidence of Dr. Mansell William Griffiths dated January 14, 2009 also contains extensive evidence pertaining to E.coli 0157:H7 and its toxins in paragraphs 42 to 48 inclusive.

- Respondent's Motion Record, Tab 1, pages 17 – 19

3. The affidavit evidence of Dr. Jeffrey Boyd Wilson dated January 15, 2009 contains evidence pertaining to E.coli 0157:H7 and its toxins in paragraphs 26 to 29 inclusive.

- Respondent's Motion Record, Tab 2, pages 237 – 238

4. The clear inference to be drawn from the evidence described above is that pasteurization renders milk safe from E.coli 0157:H7 and its toxins.

5. In October, 2009 (approximately 9 months after the trial), the International Journal of Food Microbiology accepted and published an article by Dr. Reuven Rasooly and Paula M. Do entitled "Shiga toxin is heat-stable and not inactivated by pasteurization". A copy of the article is attached to this Notice of Motion. The article describes an experiment which purported to demonstrate that even though pasteurization may kill the bacteria E.coli 0157:H7, it does not inactivate the Shiga toxin.

6. If the Rasooly/Do article is correct, the evidence of Dr. Griffiths and Dr. Wilson may have misled the court into erroneously believing that pasteurization would render raw milk safe from E.coli 0157:H7 and its toxins.

7. According to *Palmer v. The Queen* [1980] 1 S.C.R. 759, fresh evidence should be allowed when it meets these tests:

- It could not by due diligence have been adduced at trial;
- It is relevant;
- It is credible;
- If believed, it could reasonably affect the result of a trial.

8. Notice was given to counsel for the respondent by letter dated June 19, 2012 that relief of this nature would be sought in the event that leave to appeal is granted to the applicant. However, the nature of the respondent's argument on the motion for leave, as set out in the respondent's factum, makes it necessary that the court consider this present motion simultaneously with the leave motion.

THE FOLLOWING DOCUMENTARY EVIDENCE will be used in support of the motion:

1. The affidavit of Dr. Mansell William Griffiths referred to above.
2. The affidavit of Dr. Jeffrey Boyd Wilson referred to above.
3. The transcript of oral evidence of Dr. Mansell William Griffiths referred to above.

4. The attached article by Dr. Reuven Rasooly and Paula M. Do.
5. Such further and other materials as counsel may advise and this honourable court may permit.

Date: 11 July, 2012

Karen Selick, LL.B. (LSUC # 17591W)
Barrister & Solicitor,
The Canadian Constitution Foundation
110 North Front St., A-3, Suite 320,
Belleville, ON
K8P 0A6

Phone: 888-695-9105 ext. 104
Fax: 888-877-2154

To:

Ms. Shannon Chace & Mr. Daniel Huffaker,
Counsel for Her Majesty the Queen
Attorney General of Ontario
Constitutional Law Branch
4th Floor, 720 Bay Street,
Toronto, ON M7A 2S9

Phone: 416-326-4471
Fax: 416-326-4015

And To:

Registrar,
Court of Appeal for Ontario
130 Queen Street West
Toronto, Ontario M5H 2N5

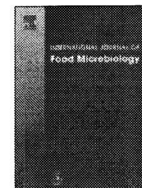
Phone:416-327-5100
Fax: 416-327-5549

International Journal of Food Microbiology 136 (2010) 290–294

Contents lists available at ScienceDirect

International Journal of Food Microbiology

journal homepage: www.elsevier.com/locate/ijfoodmicro



Shiga toxin Stx2 is heat-stable and not inactivated by pasteurization

Reuven Rasooly *, Paula M. Do

Western Regional Research Center, Agricultural Research Service, U.S. Department of Agriculture, Albany, California 94710, United States

ARTICLE INFO

Article history:
Received 4 April 2009
Received in revised form 29 September 2009
Accepted 5 October 2009

Keywords:
Shiga toxins
Escherichia coli O157:H7
Pasteurization
Vero cell
Protein inhibition

ABSTRACT

Shiga toxin-producing *Escherichia coli* have been associated with food-borne illnesses. Pasteurization is used to inhibit microbial growth in milk, and an open question is whether milk pasteurization inactivates Shiga toxins. To answer this question we measured Shiga toxin's inhibition effect on Vero cell dehydrogenase activity and protein synthesis. Our data demonstrate that Shiga toxin 2 (Stx2) is heat-stable and that pasteurization of milk, at the various suggested temperatures and times by the U.S. Food and Drug Administration, (63 °C for 30 min, or 72 °C for 15 s or 89 °C for 1 s), did not reduce the biological activity of Stx2. However, treatment at 100 °C for 5 min inactivated the toxin. These data demonstrate that Stx2 is not inactivated by conventional pasteurization.

Published by Elsevier B.V.

1. Introduction

Shiga toxigenic *Escherichia coli*, including *E. coli* O157:H7, produce a family of toxins known as Shiga toxins, or verotoxins, related to the toxin produced by *Shigella dysenteriae*. This bacterium is one of the major bacterial pathogens causing food-borne illnesses, ranging from mild diarrhea to a life threatening complication known as hemolytic uremic syndrome (Friedrich et al., 2002). It produces a family of related toxins with two major groups, Shiga toxin 1 (Stx1) and Shiga toxin 2 (Stx2). These 68 kDa toxins bind and enter eukaryotic cells via the receptor glycolipid globotriosylceramide (Gb3 and Gb4) (Lingwood et al., 1987) and cleave the N-glycosidic bond of adenine at nucleotide position 4324 in the 28s rRNA of the 60s ribosomal subunit (Endo et al., 1988), thereby inhibiting protein synthesis, resulting in cell death.

The prevalence of Shiga toxigenic group of *E. coli* (STEC) in food products of bovine origin is 16% (Madic et al., 2009). Samples of bulk tank milk from dairies across the United States suggest that 4.2% were positive for one or both Shiga toxin genes (Stx1 and Stx2) (Karns et al., 2007). Baseline data on the prevalence and characteristics of *Vero* cytotoxin-producing *E. coli* (VTEC) organisms in lactating animals in Ireland suggest ~3% of milk samples contains *E. coli* O157 (Murphy et al., 2007). In Spain ~7% of hard raw ewe's milk cheese were shown to harbor STEC isolated with the Shiga toxin stx1 gene (Caro and Garcia-Armesto, 2007). In France, the prevalence of STEC-positive samples in raw milk as determined by PCR-ELISA was 21%, of these strains, ~72% were confirmed positive for Stx (Perelle et al., 2007).

Consumption of unpasteurized raw milk and soft cheeses made from raw milk results in illnesses from STEC ranging from mild intestinal disease to severe kidney complications. Most milk consumed in these countries is pasteurized, which eliminate almost all pathogenic *E. coli*. There are no studies that show if pasteurization inactivates Shiga toxins produced by the bacteria. However an outbreak in North Cumbria in 1999, showed that haemolytic uraemic syndrome (HUS) was linked to drinking pasteurized milk, but no live bacteria was found in the milk samples (Goh et al., 2002). And the fate of the Shiga toxins in milk after pasteurization remains unclear.

Shiga toxin-producing *E. coli* has the potential to produce Shiga toxin in foods. Weeratna and Doyle (1991) have shown that inoculating *E. coli* in food will produce high levels of Shiga toxin in milk (306 ng/ml) and in meat (452 ng/ml). If ingested Shiga toxin has a potential to pose a health risk and is thus listed as a CDC select agent of biothreat. Exposure to these toxins is generally through the consumption of contaminated red meats, milk and their bi-products (Rangel et al., 2005). It has been estimated that *E. coli* serotype O157:H7 causes approximately 73,000 cases of illness per year and 61 deaths annually in the United States (Mead et al., 1999).

Sensitive, rapid and specific methods such as the polymerase chain reaction (PCR) assays have been proposed but this indirect method measures O157:H7 genes and thus does not detect toxin genes expression. Also it does not give a direct measurement of the toxin and does not distinguish between live and dead organisms. Immunoassay is not sufficient to answer this question because the immunoassay detects both active and inactive toxins. Thus, there is a need for a quantitative method to measure inactivation. The current "gold standard" test for active Shiga toxin detection measures the viability of Vero cells. This test requires several days of incubation and is not quantitative (Konowalchuk et al., 1977; Paton and Paton, 1998). To

* Corresponding author. Tel.: +1 510 559 6478; fax: +1 510 559 6429.
E-mail address: reuven.rasooly@ars.usda.gov (R. Rasooly).

0168-1605/$ – see front matter. Published by Elsevier B.V.
doi:10.1016/j.ijfoodmicro.2009.10.005

study the effect of pasteurization on the biological activity of Shiga toxin we first developed a modified bioassay using Vero cells that measured protein inhibition. To confirm our results, we also utilized a previously established method for measuring the effect of toxin on Vero cell dehydrogenase activity (Sekino et al., 2004). Both methods confirmed that in milk Stx2 is heat stable and not inactivated by pasteurization.

2. Materials and methods

Shiga toxin (Stx2) was obtained from Toxin Technology (Sarasota, FL). Human kidney cell line (HEK293) transformed with adenovirus 5 DNA (ATCC CRL-1573) and Vero cell; African Green Monkey adult kidney cells (ATCC CCL-81) were obtained from American Type Culture Collection (Manassas, VA).

2.1. Cell culture

Vero cell and HEK293 cells were maintained in Dulbecco's Modified Eagle Medium (DMEM) containing 10% fetal bovine serum and 100 units/ml of both penicillin and streptomycin were added. Cells were trypsinized when ready to harvest.

2.2. Pasteurization of milk

Milk without and with 1, 10, or 100 ng/ml Stx2 were heated in a dry bath at 63 °C for 30 min, 72 °C for 15 s, 89 °C for 1 s, and 100 °C for 5 min.

2.3. Methylthiazolyldiphenyl-tetrazolium bromide (MTT) assay

Vero cells were plated on black 96-well plates (Greiner 655090) at 1×10^4 cells in 100 µl of medium per well. The cells were incubated overnight to allow time for the cells to attach to the plate. Samples (either 5 µl of milk in 95 µl of media, or 100 µl of media spiked with Stx2) were added to separate wells and incubated for 72 h at 37 °C in a 5% CO_2 incubator. Methylthiazolyldiphenyl-tetrazolium bromide (MTT) was diluted in PBS to 2 mg/ml and 25 µl was added to each well. Plates were incubated at 37 °C for 4 h and the medium was removed. 100 µl of dimethyl sulfoxide (DMSO) was added to each well and plates were read at 540 nm. The MTT assay is a common assay for cell viability. Viable cells can reduce the yellow, water soluble MTT reagent to a purple formazan salt, which is water soluble and can be colorimetrically detected.

2.4. Generation of adenoviral vectors that express the GFP gene

To visualize the effect of Shiga toxin on living cells we measured changes in green fluorescent protein (GFP) expression levels. GFP gene was isolated from the Green Lantern vector (BRL) by digestion with the *Not I* restriction enzyme. The 750 bp fragment was purified from the gel using a Qiagen kit and was subcloned into the *Not I* site of the adenoviral shuttle plasmid between the Cytomegalovirus immediate-early promoter (CMV) and the polyadenylation signal from bovine growth hormone. As illustrated by Fig. 1 the plasmid pJM17 containing the full length of the adenovirus genome including a 4.4 kb sequence of antibiotics resistance gene were co-transfected in HEK293 cells with the shuttle plasmid containing the GFP gene flanked by the adenovirus E1 sequences. After 10 days, the cytopathic effect appeared and the transfected cells became round and detached from the plate. The cells were then analyzed by fluorescence microscopy to detect GFP gene expression. Individual plaques of Ad-GFP were amplified. The presence of the GFP gene in the virus was confirmed by Southern blot.

2.5. Preparation of high-titer viral stocks

Since most of the virus remains associated with the infected cells until very late in the infection process, high-titer viral stocks were prepared by concentrating the infected cells. Tissue culture flasks (175 mm) were seeded with HEK293 cells in DMEM containing 10% FCS and 100 units/ml penicillin/streptomycin. When cells reached 90% confluency, they were infected with the virus at a multiplicity of infection (MOI) of 10. When the cytopathic effect was nearly completed (after 48–72 h) and most of the cells were rounded but not yet detached, the cells were harvested (they were easily dislodged by tapping). Cells were pelleted by centrifugation at 800 ×*g* for 5 min at 4 °C. Both cell pellet and supernatant were collected. Since most progeny viruses remain cell-associated, infected cells were disrupted by freeze–thaw cycles followed by lysis as described below. To every cell pellet collected from 18 flasks (175 mm), 18 ml of PBS/1 mM $MgCl_2$/0.1% NP40/1 mM $CaCl_2$, were added for hypotonic lysis followed by three rounds of freeze–thawing. Crude lysates were then pelleted to remove cellular debris by centrifugation at 9500 ×*g* for 20 min at 4 °C. The supernatants that contained the crude viral lysates were carefully removed. The viral supernatants were loaded onto CsCl step gradients made by layering three densities of CsCl (1.25, 1.33, and 1.45 g/ml) and centrifuged at 50,000 ×*g* for 2 h in a Beckman SW41 rotor at 14 °C. The lower band containing the intact packaged virus was removed, and a second step density gradient of 1.33 g/ml CsCl was centrifuged for 16 h at 48,000 ×*g* at 14 °C. The lower band containing the packaged virus was collected, dialysis against 10% glycerol in saline solution. The stock viral titer, determined by plaque assays, was 10^{11} plaque-forming units/ml.

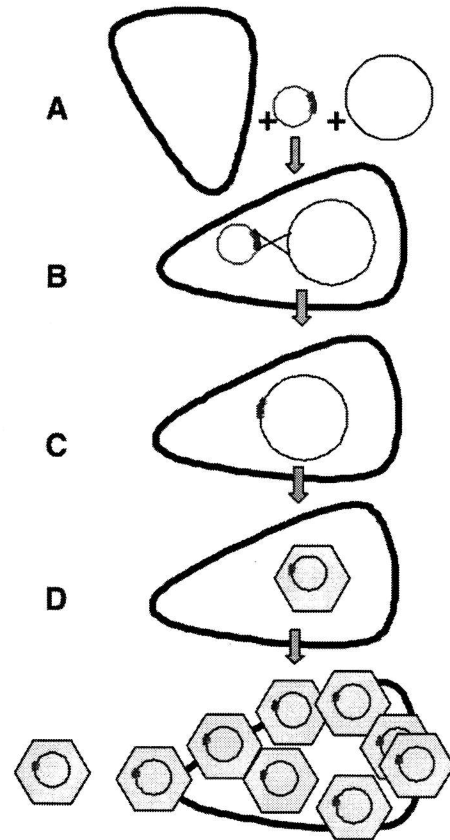


Fig. 1. A diagram illustrating the adenovirus binary cloning system. The plasmid pJM17 contains the full length sequence of the adenovirus which was co-transfected with the shuttle plasmid containing the GFP gene into HEK293 cell (A,B). This resulted in the deletion of the E1 region, including the 4.4 kb insert of the antibiotic resistance gene and the reconstruction of a full-length recombinant adenovirus genome making it a smaller size for the GFP gene to integrate into pJM17 and replace the native E1 region (C,D,). This recombinant genome was packaged, and replicated in HEK293 cells.

2.6. Plaque assays for purification and titration of the adenovirus

Plaque assays depend on the ability of the adenovirus to propagate in HEK293 cells. Six 35 mm tissue culture plates were seeded with HEK293 cells. The cells were incubated at 37 °C in a CO_2 incubator until the cells were 90% confluent. Serial dilutions (10^{-8}–10^{-13} of the adenovirus stock) were made in DMEM supplemented with 2% FBS. The diluted virus was added to the cells. After 2 h, the medium was removed and replaced with 1× Modified Eagle Medium and 1% sea-plaque agarose (FMC). The agar overlay was added to keep the virus localized after the cells had lysed. After 5 days, plaques were visible, and counted for titer determination after 7 days.

2.7. Ad-GFP assay

Vero cells were plated on black wells 96-well plates (Greiner 655090 obtained from sigma) at 1×10^4 cells in 100 µl of medium per well. Cells were incubated overnight to allow time for cells to attach to the plate and then the cells were transduced with Ad-GFP Multiplicity of Infection (MOI) of 100. After 2 h samples (either 5 µl of milk in 95 µl of media, or 100 µl of media spiked with Stx2) were added to each well and incubated for 20 h at 37 °C in a 5% CO_2 incubator. The media was removed and washed 3 times with PBS. Fluorescent emission at 528/20 nm was measured with excitation at 485/20 nm using a Synergy HT Multi-Detection Microplate Reader (BioTek, Winooki, VT).

2.8. Food matrix interference

To determine if milk affects GFP interference we incubated different volumes of milk with Vero cells transduced with GFP, and measured fluorescence emission. Milk were diluted in media and had a final volume of 100 µl (i.e. 20% of milk consisted of 20 µl of milk and 80 µl of media).

2.9. Statistical analysis

Statistical analysis was performed using SigmaStat 3.5 for Windows (Systat Software, San Jose, CA). Multiple comparisons of spiked items were made. One-way analysis of variance (ANOVA) was used to compare control media or milk with media or milk containing increasing concentrations of Shiga toxin. The experiments were repeated at least three times and results with $p < 0.05$ were considered statistically significant.

3. Results

3.1. The effects of Shiga toxin on the GFP transduced Vero cell

To examine the relationship between viral multiplicity of infection (MOI), GFP fluorescence intensity, and response to toxin, Vero cells were transduced with Ad-GFP at MOIs of 0, 1, 10^2, or 10^3, and treated with 10 ng/ml of Stx2 followed by incubation for 24, 48, and 72 h. Our results show that at MOI of 10^2, and 10^3 Shiga toxin decreased GFP fluorescence intensity (Fig. 2). As multiplicity of infection increases there is a larger difference between the fluorescence intensity of control cell and Shiga toxin treated cell.

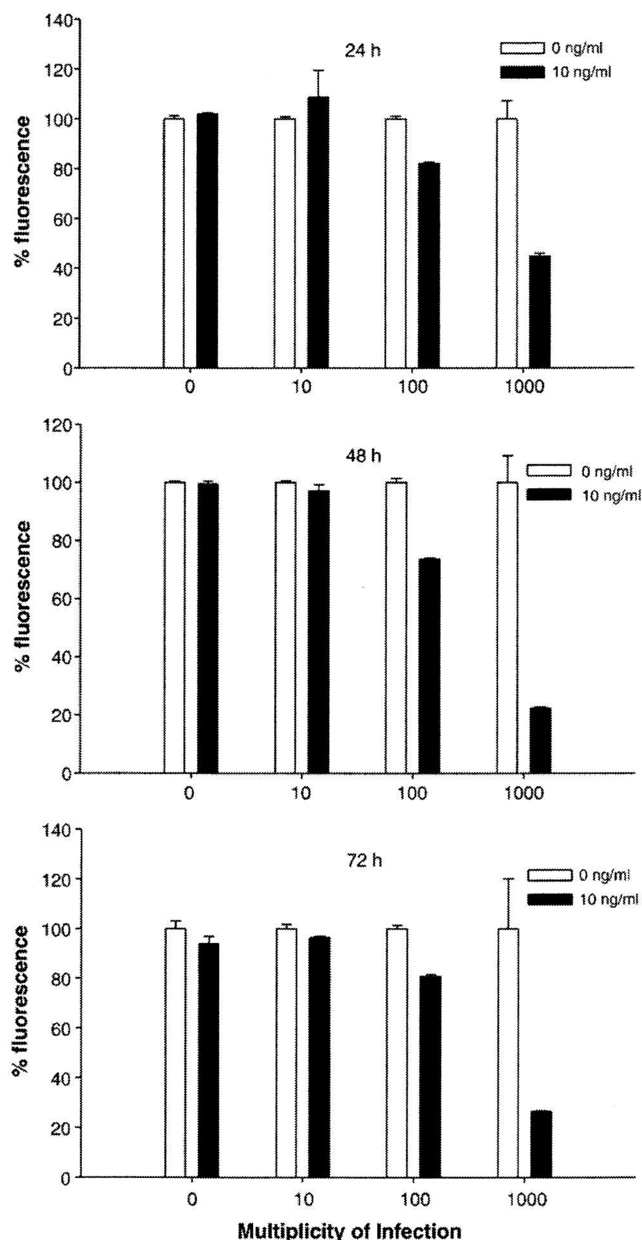


Fig. 2. Assay optimization. Vero cells were transduced with Ad-GFP at an MOI of 0, 1, 10^2, and 10^3, and treated with 10 ng/ml of Stx2 and incubated for 24, 48, and 72 h. Error bars represent standard errors, and an asterisk (*) indicates significant differences ($p < 0.05$) between spiked and unspiked media.

3.2. Validation of protein inhibition assay on Stx2

To validate the protein inhibition bioassay for measuring active Shiga toxin we fluorometrically measured inhibition of GFP expression in transduced Vero cells and compared it to measuring Vero cell dehydrogenase activity by colorimetric quantification of the accumulation of MTT-formazan formed after the reduction of MTT. As shown in Fig. 3 both bioassays showed significant differences between increasing concentrations of Stx2 ranging from 0.01 ng/ml to 100 ng/ml. The absorbance of MTT-formazan and GFP fluorescence emission decreased in a dose-dependent manner that was highly correlated with toxin concentration, with linear correlation coefficient of $R^2 = 0.928$ for MTT; $R^2 = 0.972$ for GFP. These results indicate that there is a high correlation between the amount of active Stx2, dehydrogenase activity and reduction in GFP fluorescence emission. It is assumed that the reduction in fluorescence is due to inhibition in protein synthesis.

3.3. Determination of food matrix interference

To use an *in vitro* Stx2 activity cell based assay, it is essential to study the effect of food matrices on GFP expiration. Increasing volumes of milk were added to transduced Vero cells. After incubation for 16 h we measured GFP fluorescence intensity. Our results show milk had an adverse effect on the cell (Fig. 4). There is no significant

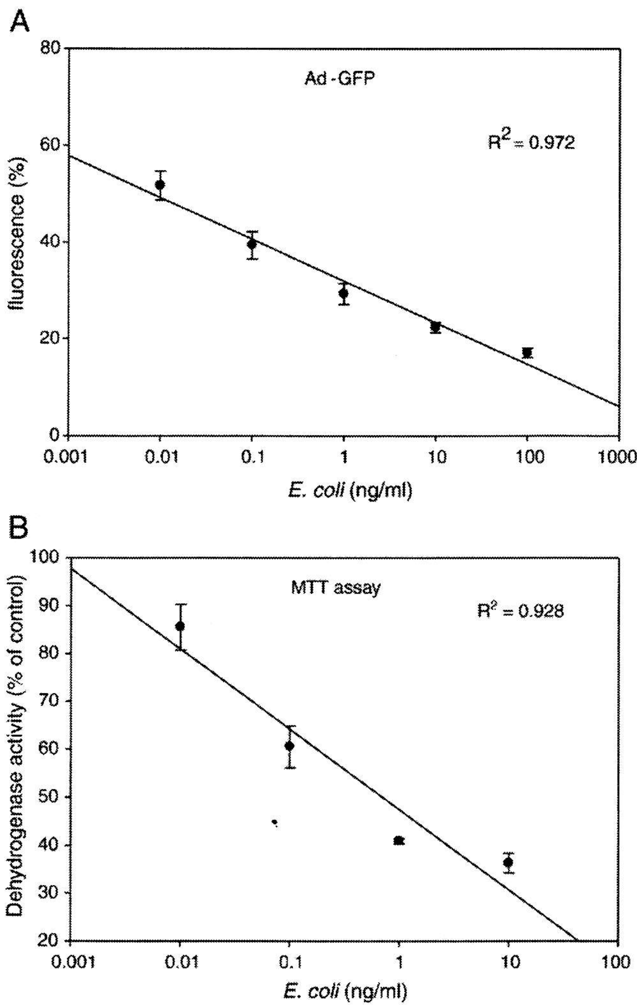


Fig. 3. Detection of Shiga toxin. Increasing concentrations of Shiga toxin were incubated with Vero cells for 18 h and inhibition of GFP expression was quantified fluorometrically (A) or dehydrogenase activity was colorimetrically measured after 72 h (B). Error bars represent standard errors.

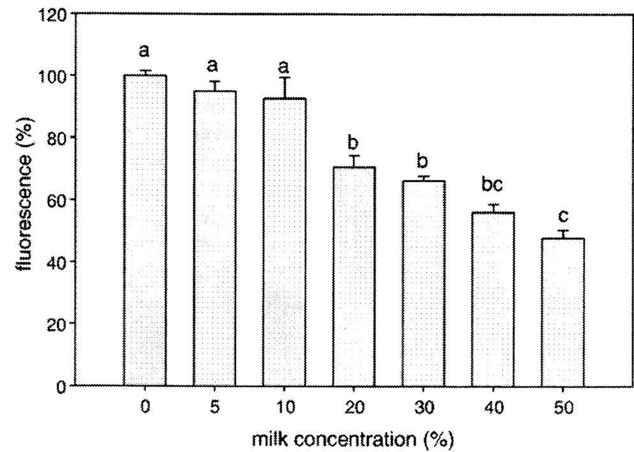


Fig. 4. Food matrix decrease GFP expiration. Vero cells were diluted with various concentration of milk to a final volume of 100 µl. After incubation for 16 h GFP expiration was measured. Error bars represent standard errors.

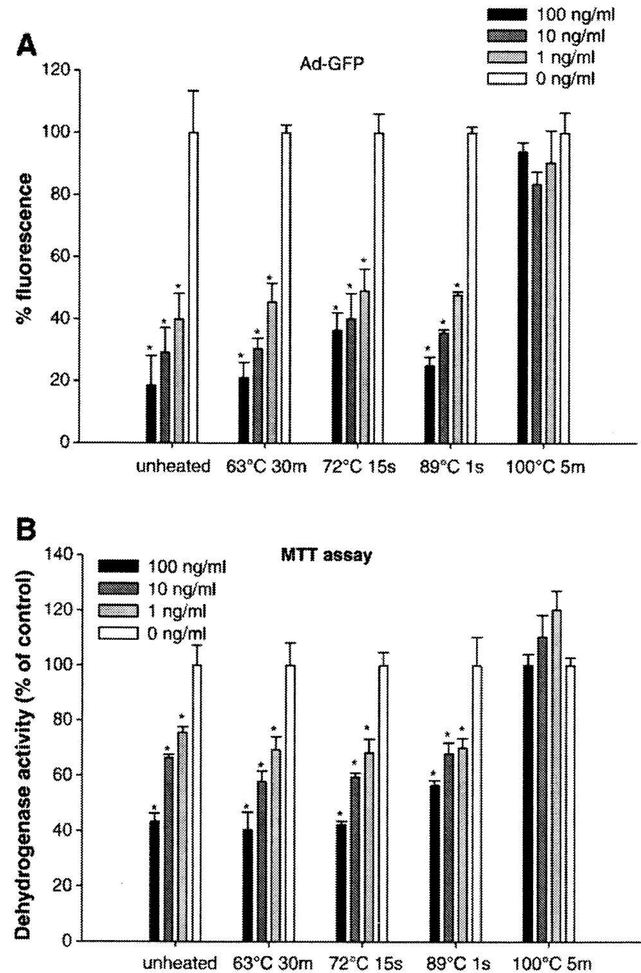


Fig. 5. Pasteurization did not reduce the biological activity of Shiga toxin. Whole milk was spiked with increasing concentrations of Shiga toxin and pasteurized at 63 °C for 30 min, 72 °C for 15 s, 89 °C for 1 s and thermal treatment at 99 °C for 5 s. Five microliters of spiked milk with 95 µl of media was added to Vero cell, after incubation for 18 h inhibition of GFP expression was quantified fluorometrically (A) or dehydrogenase activity was colorimetrically measured (B). Error bars represent standard errors, and an asterisk (*) indicates significant differences ($p < 0.05$) between spiked and unspiked milk.

difference between 5% and 10% milk from control (i.e. media); however, at >20% of milk the amount of fluorescence decreases from control. These results demonstrate that application of this assay into complex food samples will require dilution of matrix to less than 10% prior to addition to transduced Vero cells.

3.4. Thermal inactivation of Stx2

To test if pasteurization inactivates toxin we spiked whole milk with increasing concentrations of Shiga toxin and heated samples at 63 °C for 30 min, 72 °C for 15 s, 89 °C for 1 s or 100 °C for 5 min and tested Stx2 activity with GFP assay. As shown in Fig. 5, regular milk pasteurization did not reduce the biological activity of Shiga toxin; however treatment at 100 °C for 5 min inactivated the toxin. These results were confirmed by MTT assay (Fig. 5B) and suggest that standard milk pasteurization does not inactivate Shiga toxin. Our results also show that these quantitative assays can distinguish between the active and inactive form of the toxin.

4. Discussion

In this study we demonstrated that milk pasteurization does not inactivate Shiga toxin. This question was raised after an outbreak in

North Cumbria in March 1999 (Goh et al., 2002) showed an illness was strongly associated with drinking pasteurized milk from a local farm, although at the time of the outbreak no organisms from milk samples were able to grow and ferment on MacConkey agar. Study has shown that inoculating milk with *E. coli* O157:H7 will produce high levels (306 ng/ml) of Shiga toxin (Weeratna and Doyle, 1991). We hypothesized that pasteurization destroyed the bacteria, but did not affect activity of the Shiga toxin produced and secreted by the bacteria. To answer this question we first established a quantitative protein inhibition bioassay for the detection of biological active Shiga toxin in milk. This assay allows the visualization of the effect of Shiga toxin on living cells without added substrates or using cell fixation methods. When spiked milk was added to the cells the toxin enters into the cell via the highly expressed Gb3 and Gb4 Vero cell receptors. The toxin cleaves the N-glycosidic bond of adenine in the rRNA, inhibiting GFP protein synthesis thus reducing GFP fluorescence intensity in proportion to Shiga toxin concentration. As multiplicity of infection increases, expression of GFP gene increases with cell fluorescence intensity. By adding Shiga toxin, there is a difference between fluorescence intensity of control cell and toxin treated cell and the assay become more sensitive at higher MOI. This simple cell based activity assay, quantified by GFP fluorescence emission, provided rapid detection and could be easily automated. One limit of this new test is that the milk must be diluted to less than 10% which could be a problem in samples with small amounts of toxin.

We used Stx2 because it is 400 times more lethal to mice than Stx1 (Tesh et al., 1993) and is associated with high virulence in humans, although this assay could be readily adapted to detect other Stx serotypes. Our quantitative protein inhibition bioassay shows that in less than 24 h we are able to detect 1 ng/ml of toxin in milk. This sensitivity is similar to the dose that kills half of the mouse tested after 3.5 days (Tesh et al., 1993). It was previously reported that Shiga toxin 1 (Stx1), that is structurally different and less toxic than Stx2, rapidly loses its enzymatic activity when the temperature increases to over 50 °C (Brigotti et al., 2004). Our data demonstrates that pasteurization of milk at 63 °C for 30 min, 72 °C for 15 s, 89 °C for 1 s did not reduce the biological activity of Shiga toxin. The observed activity of the heated and unheated Stx2 was the same. However thermal treatment at 100 °C for 5 min inactivated the toxin. This result was confirmed by Vero cells dehydrogenase activity assay and demonstrates that the fluorescence assay can distinguish between active and inactive form of the toxin. The current recommended pasteurization condition cannot inactivate Shiga toxin and may explain outbreaks with Shiga toxins associated with drinking pasteurized milk when no live *E. coli* was found. In order to inactivate heat stable Shiga toxin a higher heat treatment is needed.

Acknowledgements

We deeply thank Daphne Tamar and Sharon Abigail for helpful discussions.

References

Brigotti, M., Carnicelli, D., Vara, A.G., 2004. Shiga toxin 1 acting on DNA in vitro is a heat stable enzyme not requiring proteolytic activation. Biochimie 86, 305–309.

Caro, I., Garcia-Armesto, M.R., 2007. Occurrence of Shiga toxin-producing *Escherichia coli* in a Spanish raw ewe's milk cheese. Int. J. Food Microbiol. 116, 410–413.

Endo, Y., Tsurugi, K., Yutsudo, T., Takeda, Y., Ogasawara, T., Igarashi, K., 1988. Site of action of a Vero toxin (VT2) from *Escherichia coli* O157:H7 and of Shiga toxin on eukaryotic ribosomes. RNA N-glycosidase activity of the toxins. Eur. J. Biochem. 171, 45–50.

Friedrich, A.W., Bielaszewska, M., Karch, H., 2002. Diagnosis of Shiga toxin-producing *Escherichia coli* infections. LaboratoriumsMedizin 26, 183.

Goh, S., et al., 2002. *E. coli* O157 phage type 21/28 outbreak in North Cumbria associated with pasteurized milk. Epidemiol. Infect. 129, 451–457.

Karns, J.S., Van Kessel, J.S., McClusky, B.J., Perdue, M.L., 2007. Incidence of *Escherichia coli* O157:H7 and *E. coli* virulence factors in US bulk tank milk as determined by polymerase chain reaction. J. Dairy Sci. 90, 3212–3219.

Konowalchuk, J., Speirs, J.I., Stavric, S., 1977. Vero response to a cytotoxin of *Escherichia coli*. Infect. Immun. 18, 775–779.

Lingwood, C.A., et al., 1987. Glycolipid binding of purified and recombinant *Escherichia coli* produced verotoxin in vitro. J. Biol. Chem. 262, 8834–8839.

Madic, J., et al., 2009. Screening of food raw materials for the presence of Shiga toxin-producing *Escherichia coli* O91:H21. Lett. Appl. Microbiol. 48, 447–451.

Mead, P.S., Slutsker, L., Griffin, P.M., Tauxe, R.V., 1999. Food-related illness and death in the United States reply to Dr. Hedberg. Emerg. Infect. Dis. 5, 841–842.

Murphy, M., et al., 2007. Surveillance of dairy production holdings supplying raw milk to the farmhouse cheese sector for *Escherichia coli* O157, O26 and O111. Zoonoses Public Health 54, 358–365.

Paton, J.C., Paton, A.W., 1998. Pathogenesis and diagnosis of Shiga toxin-producing *Escherichia coli* infections. Clin. Microbiol. Rev. 11, 450–479.

Perelle, S., Dilasser, F., Grout, J., Fach, P., 2007. Screening food raw materials for the presence of the world's most frequent clinical cases of Shiga toxin-encoding *Escherichia coli* O26, O103, O111, O145 and O157. Int. J. Food Microbiol. 113, 284–288.

Rangel, J.M., Sparling, P.H., Crowe, C., Griffin, P.M., Swerdlow, D.L., 2005. Epidemiology of *Escherichia coli* O157:H7 outbreaks, United States, 1982–2002. Emerg. Infect. Dis. 11, 603–609.

Sekino, T., et al., 2004. Characterization of a Shiga-toxin 1-resistant stock of Vero cells. Microbiol. Immunol. 48, 377–387.

Tesh, V.L., et al., 1993. Comparison of the relative toxicities of Shiga-like toxins type I and type II for mice. Infect. Immun. 61, 3392–3402.

Weeratna, R.D., Doyle, M.P., 1991. Detection and production of verotoxin 1 of *Escherichia coli* O157:H7 in food. Appl. Environ. Microbiol. 57, 2951–2955.