Heat fixation inactivates viral and bacterial pathogens and is compatible with downstream MALDI mass spectrometry tissue imaging

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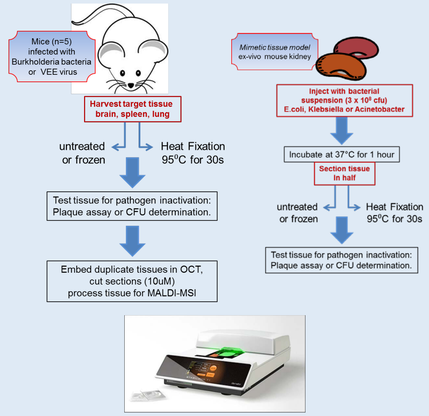
Overview: Heat fixation involves tissue stabilization utilizing a combination of heat and pressure under vacuum. This work describes the use of heat-fixation of infectious tissues, for inactivation of pathogens as well as proteomic stabilization for downstream MALDI tissue imaging.

Introduction: Standardization of sampling and rapid stabilization of molecules of interest is key to high quality tissue proteomic studies. Proteins, peptides, and their modifications can change rapidly post sampling. The Denator Stabilizor System (Gothenburg, Sweden) uses an additive-free heat-stabilization technology that utilizes conductive heating, under controlled pressure, to rapidly eliminate enzymatic degradation. Proteomic analysis of tissue for infectious disease research must overcome the additional challenge of inactivation of pathogens before downstream analysis can take place. In this work, we examined the potential of heat fixation for pathogen inactivation. In addition, duplicate heat fixed tissue samples were processed for MALDI mass spectrometry tissue imaging to evaluate the compatibility of heat fixation with this technique.

Methods: Female BALB/c mice were infected with the viral pathogen, VEE (Venezuelan Equine Encephalitis) or the bacterial pathogens, *Burkholderia thailandensis* or *B. mallei*. Five mice were infected with each pathogen. After active infection was established, the primary target organ for each pathogen was harvested: brain and spleen tissue from VEE infected mice and lung and spleen tissue from Burkholderia infected animals. Tissues were rapidly removed and heat fixed or snap frozen within 10 minutes of animal sacrifice by cervical dislocation with anesthesia.

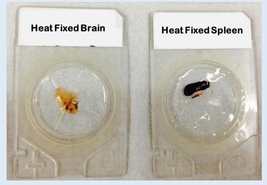
Research was conducted under an IACUC approved protocol in compliance with the Animal Welfare Act, PHS Policy, and other Federal statutes and regulations relating to animals and experiments involving animals. The facility where this research was conducted is accredited by the Association for Assessment and Accreditation of Laboratory Animal Care, International and adheres to principles stated in the Guide for the Care and Use of Laboratory Animals, National Research Council, 2011.

Methods (cont.):



Tissue samples were weighed and lysates made with a bead beater device. For the determination of viable virus from brain and spleen tissue, lysates were used in a plaque assay using Vero cells. For determination of viable bacteria, lysates from lung and spleen were tested by plating dilutions on LB or blood agar. Additional inactivation studies were conducted using a tissue mimetic model. Briefly, kidneys were harvested from control animals used in the above studies and inoculated with strains of *E.coli*, Kelbsiella and Acinetobacter ex-vivo. After incubation for 1 hour at 37°C, the kidney tissues were divided in half. One half was heat fixed and the other half was not treated. Tissue lysates were made and plated as described above. Duplicate tissue samples from the mouse infection studies were embedded in OCT freezing medium and sectioned using a cryostat. Sections (10µm) were placed on ITO coated slides, and rinsed in 70% EtOH for 30 seconds, 95% EtOH for 30s and a brief water wash to remove OCT. The slides were sprayed with SPA (10mg/ml in 50% CAN, 1% TFA) using the ImagePrep (Bruker Daltonics). MALDI-MSI was performed using an AutoFlex III (Bruker Daltonics) operated in linear mode over a mass range of 2-20kDa and the data was processed using Flex Imaging 4.0.

Methods (cont.):



Results: The presence of viable viral and bacterial pathogens was confirmed in the untreated target tissue. No viable pathogens were detected in the heat fixed tissues indicating complete inactivation.

Table 1. Plaque assay results from untreated and heat fixed VEE (vaccine strain) infected tissue.

(mg)	Dilution	10^{-1}	10^{-2}	10^{-3}	10^{-4}	Titer (pfu/ml)
100	VEE Brain untreated	100+	21	3	0	2.55E+04
		100+	30	8	0	
27	VEE Spleen untreated	0	0	0	0	0.00E+00
		0	0	0	0	
47	VEE Kidney untreated	0	0	0	0	0.00E+00
		0	0	0	0	
140	Uninfected Brain	0	0	0	0	0.00E+00
		0	0	0	0	
125	Uninfected Spleen	0	0	0	0	0.00E+00
		0	0	0	0	
140	VEE Heat Fixed Brain (1)	0	0	0	0	0.00E+00
		0	0	0	0	
55	VEE Heat Fixed Spleen (1)	0	0	0	0	0.00E+00
		0	0	0	0	
23	VEE Heat Fixed Kidney (1)	0	0	0	0	0.00E+00
		0	0	0	0	
133	VEE Heat Fixed Brain (2)	0	0	0	0	0.00E+00
		0	0	0	0	
50	VEE Heat Fixed Spleen (2)	0	0	0	0	0.00E+00
		0	0	0	0	
53	VEE Heat Fixed Kidney (2)	0	0	0	0	0.00E+00
		0	0	0	0	

Table 2. Plaque assay results from untreated and heat fixed VEE (virulent strain) infected tissue.

(mg)	Dilution	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	Titer (pfu/ml)
60	VEE Spleen untreated (1)	TNTC	TNTC	42	7	0	0	7.00E+05
50	VEE Spleen untreated (2)	45	13	0	0	0	0	1.30E+04
50	VEE Spleen untreated (3)	39	11	0	0	0	0	1.10E+04
70	VEE Spleen untreated (4)	38	9	0	0	0	0	9.00E+03
50	VEE Heat Fixed Spleen (1)	0	0	0	0	0	0	0.00E+00
60	VEE Heat Fixed Spleen (2)	0	0	0	0	0	0	0.00E+00
50	VEE Heat Fixed Spleen (3)	0	0	0	0	0	0	0.00E+00
70	VEE Heat Fixed Spleen (4)	0	0	0	0	0	0	0.00E+00
210	VEE Brain untreated (1)	TNTC	TNTC	TNTC	TNTC	TNTC	42	4.20E+08
240	VEE Brain untreated (2)	TNTC	TNTC	TNTC	TNTC	58	14	1.40E+08
220	VEE Brain untreated (3)	TNTC	TNTC	TNTC	TNTC	TNTC	37	3.70E+08
190	VEE Brain untreated (4)	TNTC	TNTC	TNTC	TNTC	TNTC	34	3.40E+08
190	VEE Heat Fixed Brain (1)	0	0	0	0	0	0	0.00E+00
180	VEE Heat Fixed Brain (2)	0	0	0	0	0	0	0.00E+00
260	VEE Heat Fixed Brain (3)	0	0	0	0	0	0	0.00E+00
210	VEE Heat Fixed Brain (4)	0	0	0	0	0	0	0.00E+00

Table 3. Results from untreated and heat fixed *B. thailandensis* and *B. mallei* infected tissue lysates.

(mg)	Bacteria (organ) (n)	10^{-1}	cfu/ml
40.3	B.t. lung untreated	1000+	$>10^3$
52.1		1000+	$>10^3$
123	B.t. Spleen untreated	70	700
128		71	710
39.5	B.t. Heat Fixed lung (1)	0	0
56.3		0	0
90.7	B.t. Heat Fixed Spleen (1)	0	0
NA		0	0
48.1	B.t. Heat Fixed lung (2)	0	0
34.4		0	0
130	B.t. Heat Fixed Spleen (2)	0	0
NA		NA	NA
	B.m. lung untreated (3)	1000+	$>10^3$
		1000+	$>10^3$
	B.m. heat fixed (3)	0	0
		0	0

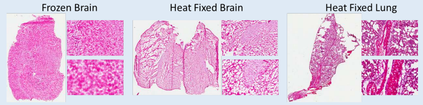
Results (cont.):

Heat fixation using the structure preserving method of 95 degrees for 30 seconds in the tissue mimetic model, resulted in no colony formation when lysates were plated on LB agar indicating complete inactivation of the bacterial pathogens present.

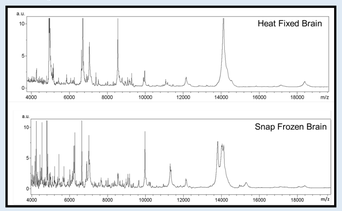
Table 4. Results from untreated and heat fixed kidney tissue collected from a tissue infection mimetic model using 6 bacterial strains

Sample Name	treatment	1:10	1:100	1:1000	cfu/ml
control	heat fixed	0	0	0	0
E.coli Top10	heat fixed	0	0	0	0
E.coli pGSV3-GmR	heat fixed	0	0	0	0
E.coli pAM17-KanR	heat fixed	0	0	0	0
Klebsiella pneumoniae	heat fixed	0	0	0	0
Burkholderia thailandiensis	heat fixed	0	0	0	0
Acinetobacter baumani	heat fixed	0	0	0	0
control (no bacteria)	null	0	0	0	0
E.coli Top10	null	TNTC	1000+	1000+	$>10^3$
E.coli pGSV3-GmR	null	TNTC	1000+	1000+	$>10^3$
E.coli pAM17-KanR	null	TNTC	1000+	1000+	$>10^3$
Klebsiella pneumoniae	null	TNTC	1000+	1000+	$>10^3$
Burkholderia thailandiensis	null	TNTC	1000+	1000+	$>10^3$
Acinetobacter baumani	null	TNTC	1000+	1000+	$>10^3$

Hemotoxylin and Eosin stained sections reveal preservation of the major structures in the tissue, but changes to the fine structure were observed at the cellular level.

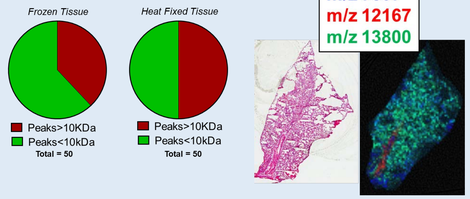


Parallel samples were prepared on slides for MALDI tissue imaging after heat fixation by embedding in OCT and freezing. The effect of heat stabilization on the tissue increased rigidity, and the tissue appeared darker and slightly reduced in size. Changes to the fine structure seen in heat fixed tissue did not appear to adversely affect our ability to obtain good quality spectra for MALDI-MSI.

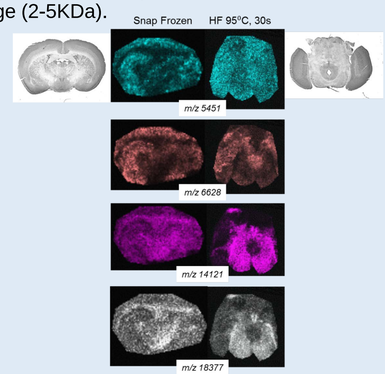


Results (cont.)

Spectra from heat fixed brain tissue exhibited fewer low molecular weight peaks which may be indicative of protein degradation products.



MALDI-MSI of the heat fixed tissue produced images with greater peak intensities in the higher mass range (12-20kDa), and fewer low molecular weight degradation products visible in the low mass range (2-5KDa).



Conclusion: Heat Fixation inactivated both bacterial and viral pathogens in mouse tissue.

Heat fixed tissue can be embedded in OCT and sectioned on a cryostat for MALDI-MSI imaging.

Heat Fixation preserves tissue for proteomic analysis via MALDI-MSI imaging.

Opinions, interpretations, conclusions, and recommendations are those of the author and are not necessarily endorsed by the U.S. Army.

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