MendelNet

Editors:

Radim Cerkal

Natálie Březinová Belcredi

Lenka Prokešová

Proceedings of 25th
International PhD Students Conference

7-8 November 2018, Brno, Czech Republic

Preparation of cryosections from frozen porcine pulmonary tissue for MALDI mass spectrometry imaging

Rea Jarosova[1], Vendula Smolikova[2,3], Marek Dvorak[2], Roman Guran[2,3], Tomas Do[2], Petra Ondrackova[4], Ondrej Zitka[2,3], Zbysek Sladek[1]
[1]Department of Morphology, Physiology and Animal Genetics
[2]Department of Chemistry and Biochemistry
Mendel University in Brno
Zemedelska 1, 613 00 Brno
[3]Central European Institute of Technology
Brno University of Technology
Purkynova 123, 612 00 Brno
[4]Department of Immunology
Veterinary Research Institute
Hudcova 296/70, 621 00 Brno
CZECH REPUBLIC
xjaroso3@node.mendelu.cz

Abstract: In last decades, a matrix-assisted laser desorption/ionization mass spectrometry imaging (MALDI MSI) was used for mapping the spatial distribution of different molecules, mainly peptides, proteins, lipids and metabolites, in different types of tissue. Cryosections are the best suited for getting as much information as possible because the preparation of samples for MALDI MSI does not include deparaffinization and antigen-retrieval steps which are needed in case of conventional formalin-fixed and paraffin-embedded (FFPE) tissue sections. To analyse infection markers like interleukins in the tissue sections of porcine lungs affected by *Actinobacillus pleuropneumoniae*, it was necessary to obtain very well prepared cryosections for MALDI MSI. Therefore, this work was focused on optimization of preparation of frozen porcine lungs and its cryosections with conserved morphological structure.

Key Words: cryosectioning, lungs, pig, pleuropneumonia, mass spectrometry imaging

INTRODUCTION

The *Actinobacillus pleuropneumoniae* (APP) bacterium causes pleuropneumonia in pigs, which, together with the porcine reproductive and respiratory syndrome (PPRS), is the most widespread disease in swine-breeding, where it causes significant breeding and economic losses (Zimmerman et al. 2012). This Gram-negative bacterium colonizes the cells of the lower respiratory tract (Baarsch et al. 2000), where it causes necrotic and hemorrhagic pneumonia (Zimmerman et al. 2012) and thus induces an inflammatory response in the lungs of pigs (Inzana 1991). APP infection is therefore a suitable model for the study of an inflammatory response.

Specific patterns of acute and chronic inflammation are seen during particular situations that arise in the body. Therefore, detection of pathological changes in tissues may indicate the stage of the inflammatory process. However, this method reveals only those processes that are visible. Beside this, cell-derived mediators, plasma-derived mediators, high systemic levels of acute-phase proteins and other agents are released during inflammation. It is therefore obvious that the use of analytical methods for the detection of inflammatory components provides essential data for understanding the pathogenesis. The use of new methods for detection of inflammation components is therefore highly relevant.

Matrix-assisted laser desorption/ionization mass spectrometry (MALDI MS) has been applied to the analysis of biomolecules (DNA, proteins, peptides and sugars) and large organic molecules. Therefore, MALDI is a diagnostic tool with much potential because it allows the rapid identification of proteins and changes to proteins without the cost or computing power of sequencing. MALDI is used for mass spectrometry imaging (MSI), providing the information about the spatial distribution of

molecules in various tissues (Stoeckli et al. 2001). General workflow of MALDI MSI includes collecting thin tissue sections using a cryostat (Tucker et al. 2011), attaching sections on a sample plate or ITO (indium-tin oxide) glass slide, and depositing of a matrix (Rohner et al. 2005). Besides cryo-sectioned frozen tissues, formalin-fixed and paraffin-embedded (FFPE) tissues may be used for MALDI MSI, but FFPE tissue demands more complicated preparation prior to MSI analysis because paraffin can have negative effect on ionization and formaldehyde fixation causes dehydration, denaturation, crosslinking (methylene bridges), precipitation and protein agglutination, which have negative influence on detection (Guráň et al. 2016).

The aim of this work was the optimization of sectioning of frozen porcine lungs to produce cryosections of the best quality for the MALDI MSI.

MATERIAL AND METHODS

Chemicals and material

All solvents (HPLC grade) and other chemicals were purchased from Sigma Aldrich (St. Louis, MO, USA), unless otherwise stated. Conductive indium-tin oxide (ITO) one-side coated glass slides and peptide/protein calibration standards were purchased from Bruker Daltonik GmbH (Bremen, Germany).

Animals and experimental infection

In this experiment were used 4 eight-week-old pigs (*Sus scrofa f. domestica*) from the herd without APP anomaly or vaccination. The pigs were kept in the accredited barrier-type animal facilities of the Veterinary Research Institute (VRI). The animal care protocol for this experiment followed the Czech guidelines for animal experimentation and was approved by the Branch Commission for Animal Welfare of the Ministry of Agriculture of the Czech Republic. The pigs were allowed to acclimatize in the animal facilities for one week, and then the experimental infection was performed.

An The infection with APP field strain (biotype 1, serotype 9, KL2–2000) from the fourth passage was performed intranasally during inhalation, and the infectious dose of 1×10^9, 1×10^8, 1×10^7, 1×10^6 bacteria for each individually pig was administered to the second third of each nasal cavity of pig. The total infective dose of 4 ml was administered 2 ml per nasal cavity. Infected pigs were euthanized 24 h after infection. The experimental materials - porcine lungs and tracheobronchial lymph node (TBLN) were collected by trained staff from authorized and registered slaughterhouse at VRI.

Preparing of frozen sections

Porcine necrotic lung tissue was filled over the bronchus by mixture of embedding medium Tissue Tek (O.C.T. compound, Sacura – Finnetek) with phosphate-buffered saline solution (PBS) (Bio Whittaker, Lonza) in rate 1 : 1 for preserving a morphological structure of lungs compared to physiology state. TBLN were inserted into the embedding medium Tissue Tek, and all samples were frozen by super-cooled n-heptane placed on the dry ice.

The tissue samples were cut to a thickness of 5–10 μm using the cryostat (Leica Microsystems, CM 1900, GmbH, Wetzlar, Germany) at temperature -20 °C (Figure 1). The cuts of tissue were placed on slides and fixed in pre-cooled acetone for 5 minutes and stained with Mayer haematoxylin. The histological slides were evaluated by using the light microscope (Olympus BH-2, Japan) and images were taken by camera (Canon EOS 1100D, Japan) using software (Quick Photo Micro 3.0, PROMICRA, Czech Republic).

Preparing of frozen sections for MALDI MSI

Porcine necrotic lung tissue was filled over the bronchus by mixture of embedding medium Tissue Tek (O.C.T. compound, Sacura-Finnetek) with phosphate-buffered saline solution (PBS) (Bio Whittaker, Lonza) in rate 1 : 1 for preserving a morphological structure of lungs compared to physiology state. TBLN were inserted into the embedding medium Tissue Tek, and all samples were frozen by super-cooled n-heptane placed on the dry ice.

The tissue samples were cut to a thickness of 7, 8 and 10 μm on the cryostat (Leica Microsystems, CM 1900, GmbH, Wetzlar, Germany) at temperature -20 °C. The cuts of tissue were placed on ITO slides and fixed in 70% ethanol for 90 sec, then 100% ethanol for 90 s, ten times soaked in ultra-distilled water and again fixed in 70% ethanol for 90 s, then 100% ethanol for 90 s. Slides were stored at -80 °C.

Figure 1 The Leica CM1900 Cryostat –this apparatus was used to section frozen tissue onto slides

MALDI MSI analysis

The MSI was performed on a MALDI-TOF mass spectrometer Bruker ultrafleXtreme (Bruker Daltonik GmbH, Bremen, Germany) using a protocol according to Guran and coworkers (Guran et al. 2017). The total sample set consisted of 2 ITO glass slides containing 4 tissue sections. MALDI matrix was sprayed onto ITO glass slides using ImagePrep™ standard program (Bruker Daltonik GmbH, Bremen, Germany). Sinapinic acid (SA) was used as MALDI matrix. SA was prepared in a concentration of 10 mg/ml in 60% acetonitrile and 0.2% trifluoroacetic acid (TFA). The scanned images of tissue slices were loaded into FlexImaging 3.0 software (Bruker Daltonik GmbH, Germany), and a MALDI adapter with two ITO glass slides was loaded into the mass spectrometer. The position of the MALDI adapter was adjusted according to the white guide marks on the ITO glass slides. The regions of acquisition were highlighted by the mouse pointer in FlexImaging, and 50 μm width of raster spot was chosen. External calibration was performed using a protein standard mixture in an *m/z* range of 4–20 kDa. The MSI images were generated and visualized using SCiLS Lab 2014b software (SCiLS–Bruker Daltonik GmbH, Bremen, Germany). The laser power was set to 85%. MALDI MSI of proteins was performed in linear positive mode in a *m/z* range of 4–20 kDa. A total of 1000 spectra were summed for each spot.

RESULTS AND DISCUSSION

The data obtained from our histological sections showed, that the most suitable method of processing pulmonary tissue samples was filling the lungs by cryoprotective agent for preserving the morphological structure and freeze it in a super-cooled n-heptane. In comparison to the authors (Prince and Porter 1975), whose used a 1 : 2 mixture of an O.C.T. embedding compound (Tissue Tek) and phosphate-buffered saline (PBS) injected intratracheally into fresh lung tissue, we have filled lungs over the lung *bronchus* by a mixture 1 : 1 of an embedding compound Tissue Tek with PBS according to Yeh and coworkers (Yeh et al. 2015) for preserving the morphological structure of the lungs compared to the physiology state. This method was used to create standard frozen sections without significant artifacts as is presented in Figures 2A, 3 and 4. Figure 2B shows unpreserved structure.

In closer look onto Figures 2A, 3 and 4, it is apparent, that all tissue structures remained almost untouched. Only the section of pulmonary tissue, which was frozen without previous filling with mixture of Tissue Tek and PBS, showed some changes; the most visible are collapsed alveoli (Figure 2B, in the bottom right of the figure). Therefore, the use of filling before freezing was necessary to make cryosections for MALDI MSI in the best quality. In scanned images of pulmonary tissue cryosections (Figure 5, top row) there are not visible any significant defects of the tissue. It confirms, that the sections were prepared as well as in Figure 3.

Figure 2 A porcine lung tissue sample filled over the lung bronchus by a mixture of Tissue Tek with PBS (1 : 1) and frozen in n-heptane placed on a dry ice. (A) preserved alveolar structure. (B) pulmonary tissue without filling with mixture of Tissue Tek with PBS, collapsed alveoli, shot structure. It does not correspond to the structure of the lungs of the physiology state. Stained by Mayer haematoxylin. In magnification 100 x
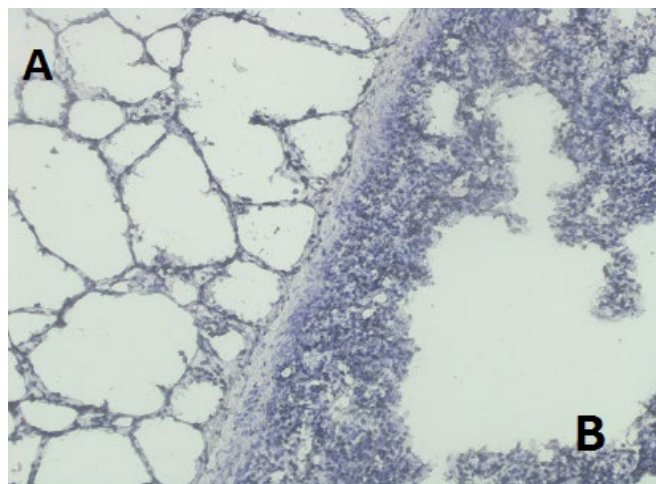

Figure 3 Porcine lung tissue frozen by n-heptane placed on a dry ice, tissue filled with mixture of Tissue Tek with PBS (1 : 1). Stained by Mayer haematoxylin. In magnification 100 x
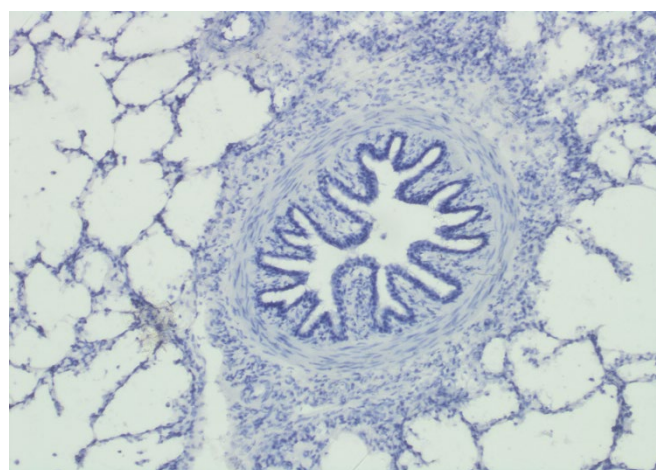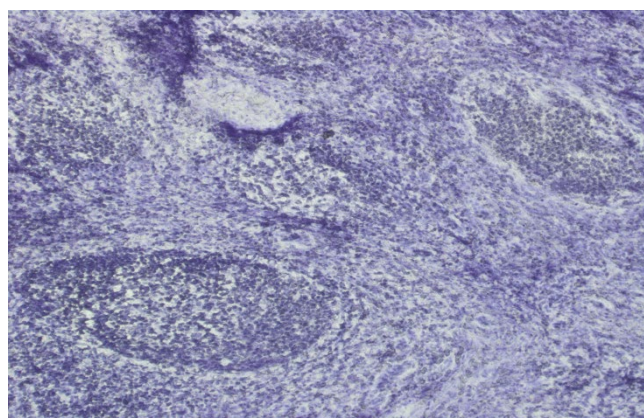

Figure 4 Porcine tracheobronchial lymph node frozen by n-heptane placed on a dry ice. Stained by Mayer haematoxylin. In magnification 100 x

The MALDI MSI analysis of prepared cryosections showed good spatial distribution of a mass peak at *m/z* 10827.5 Da (Figure 5, bottom row). This peak probably corresponds to S100 calcium-binding protein A8 (S100A8) as was investigated by Wehder and co-workers (Wehder et al.

2010). S100A8 protein is involved, inter alia, in the regulation of cell proliferation and acute inflammation (Wehder et al. 2010). Regarding the tissue slice thickness, the data from MSI provided evidence, that the highest intensity of mass peak at *m/z* 10827.5 Da was achieved at slice with 10 μm thickness (Figure 5, the first MSI image from the left in the bottom row). This was probably caused by higher dilution and diffusion of analyte at lower slice thicknesses after application of matrix solution. Based on Figure 5 it is apparent, that the process of cryosectioning of frozen porcine lungs was well optimized and therefore can be used for future experiments aiming at the detection of spatial distribution of different infection markers in porcine lungs infected by *Actinobacillus pleuropneumoniae* (APP) bacterium.

Figure 5 MALDI MSI image of mass peak at m/z 10827.5 Da in porcine lung tissue slices with thickness 10, 8 and 7 μm.
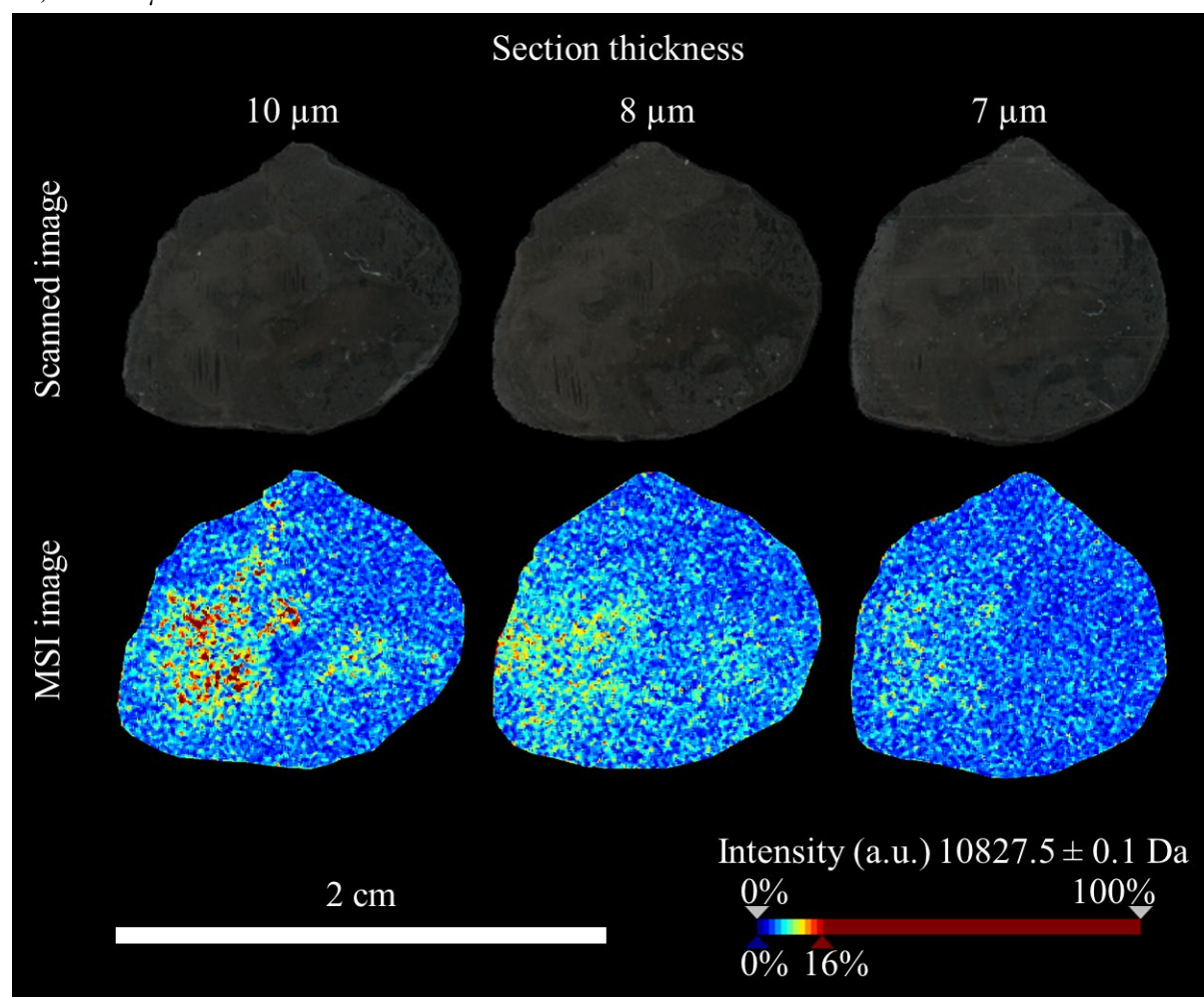
CONCLUSION

In this work, different ways of preparation of porcine lung tissue cryosections were tested. The way when lungs were filled over the lung *bronchus* by a mixture of Tissue Tek with PBS (1 : 1) and frozen in n-heptane placed on a dry ice was chosen as the most appropriate method for the subsequent evaluation by MALDI MSI.

REFERENCES

Baarsch, M.J. et al. 2000. Pathophysiologic correlates of acute porcine pleuropneumonia. American Journal of Veterinary Research, 61(6): 684–90.

Guran, R. et al. 2017. MALDI MSI of MeLiM melanoma: Searching for differences in protein profiles. PLOS ONE, 12(12): e0189305.

Guráň, R. et al. 2016. MALDI zobrazovací hmotnostní spektrometrie pro studium fyziologických pochodů v nádorech. Chemické listy, 110(2): 106–111.

Inzana, T.J. 1991. Virulence properties of Actinobacillus pleuropneumoniae. Microbial Pathogenesis, 11(5): 305–16.

Prince, G.A., Porter, D.D. 1975. Cryostat Microtomy of Lung Tissue in an Expanded State. Stain Technology, 50(1): 43–45.

Rohner, T.C. et al. 2005. MALDI mass spectrometric imaging of biological tissue sections. Mechanisms of Ageing and Development, 126(1): 177–185.

Stoeckli, M. et al. 2001. Imaging mass spectrometry: A new technology for the analysis of protein expression in mammalian tissues. Nature Medicine, 7: 493.

Tucker, K.R. et al. 2011. The modified-bead stretched sample method: Development and application to MALDI-MS imaging of protein localization in the spinal cord. Chemical Science, 2(4): 785–795.

Wehder, L. et al. 2010. Depicting the Spatial Distribution of Proteins in Human Tumor Tissue Combining SELDI and MALDI Imaging and Immunohistochemistry. Journal of Histochemistry and Cytochemistry, 58(10): 929–937.

Yeh, Y.C. et al. 2015. Using frozen section to identify histological patterns in stage I lung adenocarcinoma of </= 3 cm: accuracy and interobserver agreement. Histopathology, 66(7): 922–38.

Zimmerman, J.J. et al. 2012. Diseases of Swine. 10th ed., Wiley-Blackwell.

Contributions are published in original version, without any language correction.