Influence of Inactivation Methods on Pathogen Diagnostics by means of Instrumental Methods

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Abstract

Mass spectrometry as an identification method for microorganisms is rapidly developing in the last years. However, this method is not suitable for detection of agents in complex matrices and it has to be preceded by clean-up procedures and particular agents concentration. In case of high-risk pathogens, such a separation methods may pose hazard for the laboratory staff. Therefore, various methods for pathogen inactivation were studied. Their influence on pre-concentration and separation of microorganisms by means of preparative and capillary isoelectric focusation was studied. Most of the disinfectant agents disrupted the cells integrity and made their following separation impossible. The most promising was freeze-dry samples inactivation using hydrogen peroxide vapour at 300 ppm concentration. Inactivation of the lyophilized bacterial agents caused only inconclusive shift of mass spectras in MALDI-TOF MS analysis, but it caused quite significant change of isoelectric point. Inactivation of bacterial spores required at least 2 hours of exposure. Increased vapour concentration caused damage of the cells. Heating up the samples up to 60 °C enabled to increase the vapour concentration and made the inactivation faster without influencing the mass spectras. Moreover, the influence of thermal inactivation of bacterial agents at 60 °C for 16 hours was studied. There was no significant change in mass spectras in MALDI-TOF MS analysis, but it did not work for sporulating bacterias. Simultaneously, the long time of inactivation was a significant drawback. The possibilities of the detection and the identification of inactivated pathogens are an object of ongoing research.

Keywords: *preparative and capillary isoelectric focusing, MALDI-TOF MS, whole cells, pathogen inactivation, identification*.

Introduction

Increasing threats of bioterrorist attacks have force the need to develop methods for rapid detection and identification of biological agents. These identification methods should provide the possibility of rapid confirmation of presence of dangerous substances in order to choose the most effective methods for protecting public health.

There are various methods which can be used for identification of biologic agents and they must satisfy many criteria including sensitivity, specificity, reliability, time consumption, low risk for the laboratory staff and many others. The identification methods covers molecular biology methods, such as PCR (polymerase chain reaction), immunochemic or serologic methods as well as purification and separation methods followed by mass spectrometry. The purification process usually covers pre-concentration, clean-up procedures and separation techninques which are time-consuming and demanding on various laboratory equipment. Mass spectrometry MALDI-TOF (matrix assisted laser desorption/ionization – time of flight) as an identification method for biological agents is rapidly developing in the last years. This method is rapid and reliable, but it is not suitable for detection of agents in complex samples so the sample has to be separated using cultivation where selected unique colonies are identified. Or, the identification analysis of the complex sample has to be preceded by clean-up procedures and particular agents concentration. In case of high-risk pathogens, such separation methods may pose hazard for the laboratory staff.

Therefore, it would be worthy to inactivate the agents prior to the detection and identification of the potentially hazardous biologic agents in order to eliminate the risk for laboratory personnel. However, many of the inactivation procedures disable the detection and identification by common methods, so we searched for such inactivation procedures which would enable subsequent reliable and fast detection and identification of the B-agents.

Various methods for pathogen inactivation were considered. We focused on inactivation methods using elevated temperature and/or hydrogen peroxide vapours at various concentrations and combinations thereof. Lots of the biologic agents are sensitive to the elevated temperature, but the sporulating agents as *Bacillus anthracis*, bacterium that causes anthrax, are always difficult issue due to high resistance of the spores. Hydrogen peroxide has been shown to inactivate a wide variety of infective biological agents ranging from both vegetative cells and spores of bacteria and fungi, protozoa and their cysts, viruses and even prions [1]. There is evidence that the hydrogen peroxide vapour is capable of performing more intensive oxidation of the biological macromolecules than do the aqueous solutions of hydrogen peroxide [1]. Using of hydrogen peroxide vapours at 5-10 ppm concentration for inactivation of bacterial spores of *Bacillus anthracis* within few days under environmental conditions as a simple decontamination approach has been published recently [2]. However, this time-consuming approach is not suitable for laboratories where rapid identification of unknown sample is demanded.

Obviously, the cultivation of the inactivated microorganisms with negative result must prove the efficiency of the inactivation procedure, besides the consequent identification of the inactivated agent should be possible and reliable. Many decontamination or inactivation methods makes the identification methods impossible, so we focused on developing effective but sensitive inactivation method and combine it with suitable purification method in order to achieve inactivated B-agents evincing the same identification signs as the original viable agent.

Capillary electromigration techniques are valuable tool for separation of bioparticles which may be used for purification and clean-up procedure before the MALDI-TOF analysis. We studied possibility of using capillary electromigration techniques (CIEF – capillary isoelectric focusation and preparative IEF isolectric focusation) for separation of the bioparticles and the influence of the inactivation procedures on the bioparticle mobility and their isoelectric point.

Experimental

We used vaccination strain of *Bacillus anthracis* as a model sporulating biologic agent and non-pathogenic strain of *Eschericha coli* as a model non-sporulating biologic agent. The cells of *B. anthracis* were cultivated on special cultivation media adjusted for spore forming and the resulting cultivated cultures of *B. anthracis* and *E. coli* were lyophilized and divided in ten aliquote portions.

The inactivation chamber consisted of sealable plastic box of 25 litres volume where 150 mL of 60% hydrogen peroxide distributed in 6 small flat vessels placed on the bottom and covered with nylone membrane (Nytran® N, 0.2 µm, Whatman) to ensure low and disperse concentration the hydrogen peroxide vapours. The inactivation box was inserted into thermostat set for 20 °C, resp. 60 °C. The concentration of hydrogen peroxide vapours was maintained at 300 ppm as measured by Polytron 7000 instrument with DrägerSensor H_2O_2. Above the vessels, the sample holder was placed. The aliquote portions of the lyophilized samples were distributed into ten separate cell-culture multi-well plates which were inserted into the inactivation box. The plates with lyophilized samples were taken away step-by-step in 20 minutes interval, resp. 10 minutes interval and the inactivated samples were subjected to re-cultivation on cultivation media. In next experiment, the volume of hydrogen peroxide inside the inactivation chamber was increased to achieve concentration 600 ppm of hydrogen peroxide, and the samples were taken away in 5 minutes interval.

The time required for inactivation of the lyophilized samples is presented in table 1.

Table 1: Inactivation time at different temperatures and hydrogen peroxide concentrations

Concentration H_2O_2	Temperature	Time of inactivation
300 ppm	20 °C	120 min
300 ppm	60 °C	30 min
600 ppm	60 °C	15 min
-	60 °C	insufficient (over 16 h)

Results and Discussion

Inactivation using elevated temperature only (60 °C, without hydrogen peroxide vapours) for more than 16 h was successful for non-sporulating agent (*E. coli*), however the sporulating agents (*B. anthracis*) were not inactivated to the total, though the identification signs were kept enabling following detection and identification.

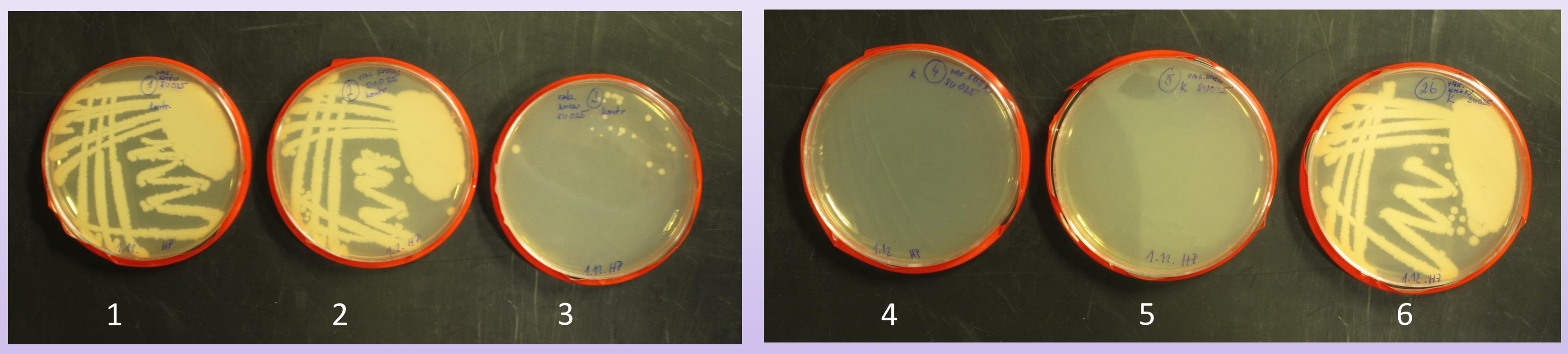
Inactivation of the lyophilized samples using hydrogen peroxide vapours at 300 ppm concentration and at 20 °C was much more promising. This procedure inactivated the biologic agents including the bacterial spores which required at least 120 min of the process, as demonstrated on **figure 1**. The MALDI-TOF mass spectra did not show any significant differences between inactivated and viable agent. However, the isoelectric point as one on the identification characteristics for isoelectric focusation changed significantly.

Increasing the concentration of hydrogen peroxide vapours up to 600 ppm caused disruption of the biologic agents and their detection and identification was then impossible.

Better results were achieved, when the temperature in the inactivation chamber was increased up to 60 °C and the hydrogen peroxide vapours concentration was set to 300 ppm – the samples were inactivated in 30 minutes and their identification characteristics for PCR analysis were maintained, as demonstrated on **figure 2**. Increasing the hydrogen peroxide concentration to 600 ppm significantly made the inactivation even faster – within 15 minutes – and the identification characteristics for PCR analysis were maintained also, as demonstrated on **figure 2**. It shows that increase of *B. anthracis* PCR products were obtained for positive control as well as for samples inactivated by H_2O_2+elevated temperature, by contrast, the samples inactivated by common bleach did were so disrupted that the subsequent PCR analysis did not show any response.

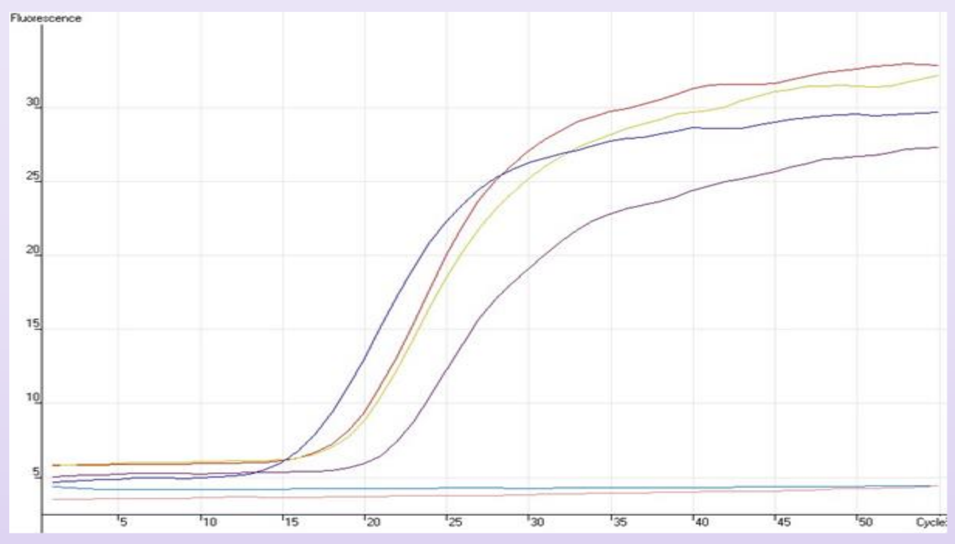
The inactivated samples were tested using molecular-biological method (PCR) and instrumental methods (MALDI-TOF and CIEF) and the identification characteristics of the inactivated samples were compared to the identification characteristics of original (non-inactivated viable) samples, as shown on **figure 2** and **figure 3**.

Figure 1: Inactivation of lyophilized *B.anthracis* at 20 °C and 300 ppm hydrogen peroxide vapours – subsequent cultivation on special agar base



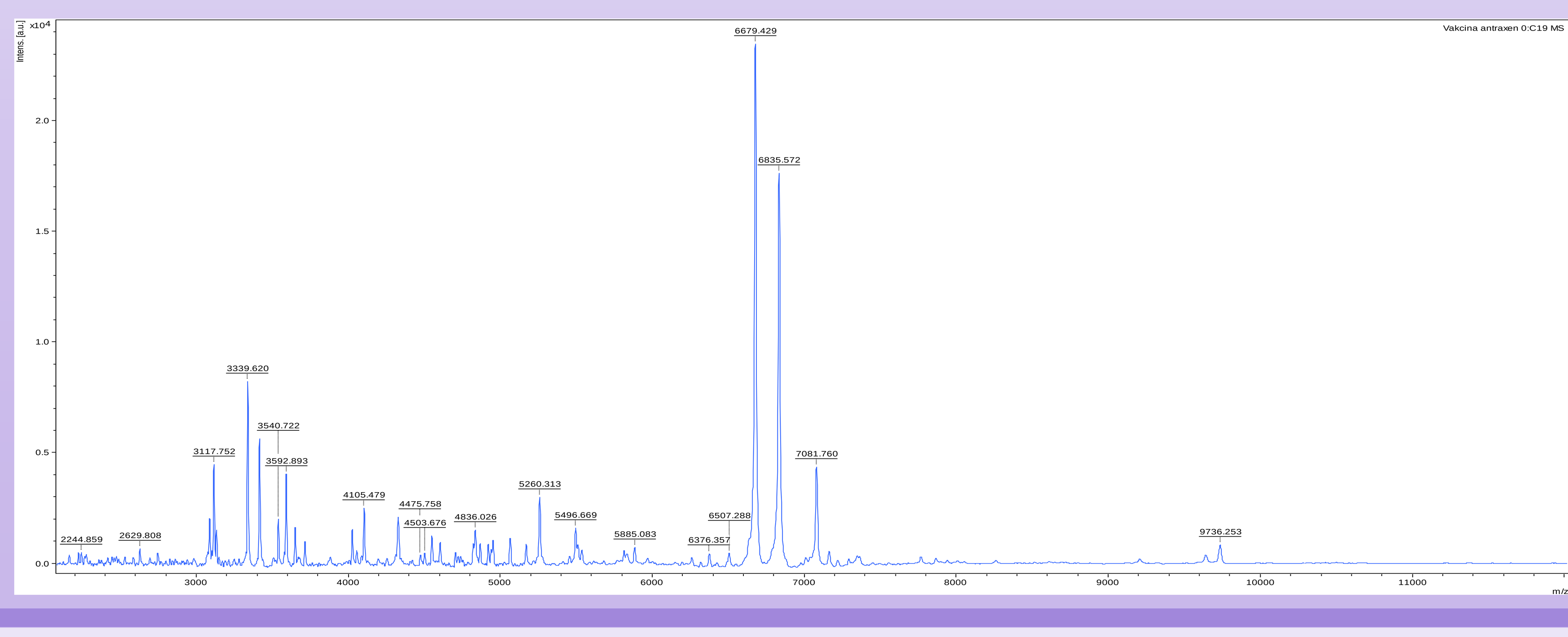
1. Original lyophilized sample without inactivation
2. Lyophilized sample after 30 min of inactivation
3. Lyophilized sample after 60 min of inactivation
4. Lyophilized sample after 90 min of inactivation
5. Lyophilized sample after 120 min of inactivation
6. Control – viable *B. anthracis* sample

Figure 2: qPCR of *B.anthracis* after various types of inactivation.



- Positive control – viable *B. anthracis*
- Inactivated *B. anthracis* – 120 min at 20 °C and 300 ppm H_2O_2
- Inactivated *B. anthracis* – 30 min at 60 °C and 300 ppm H_2O_2
- Inactivated *B. anthracis* – 15 min at 60 °C and 600 ppm
- Inactivated *B. anthracis* by common bleach
- Negative control – water

Figure 3: MALDI-TOF spectras of *B. anthracis* were without any significant changes between the viable samples and samples inactivated using elevated temperature (60 °C) and hydrogen peroxide vapours (300 ppm or 600 ppm)



Conclusion

Lyophilized biologic agents – sporulating and non-sporulating bacteria – may be inactivated effectively and rapidly using hydrogen peroxide vapours (300–600 ppm) at elevated temperature (60 °C) without significant restraints of their subsequent detection and identification using PCR or MALDI-TOF methods. Within analysis using CIEF, the shift of isoelectric points was revealed. However, the shift was stable for different modes of H_2O_2 + elevated temperature inactivation what makes the this inactivation method usable for separation of the inactivated samples supposing the database of relevant isoelectric points would be created. The elevated temperature (60 °C) inside the inactivation chamber is important, as it ensures the vapour state of the hydrogen peroxide. Whereas, at lower temperatures (20 °C), the hydrogen peroxide vapours tends to condense and the liquid state seems to disrupt the cells what makes the subsequent identification of the B-agent impossible.

References

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