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SCIENCE DIRECT
Journal of Microbiological Methods 65 (2006) 525–534
Journal of Microbiological Methods
www.elsevier.com/locate/jmicmeth

Dynamics of boar semen motility inhibition as a semi-quantitative measurement of *Bacillus cereus* emetic toxin (Cereulide)

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Received 18 May 2005; received in revised form 2 September 2005; accepted 19 September 2005
Available online 21 November 2005

Abstract

Qualitative and quantitative application of a computer assisted sperm analyzer (CASA) for detection and quantification of cereulide was described. The plot of the decrease of the percentage of boar semen progressive motility (PMOT%) in function of time and the visual inspection of curves provided a qualitative comparison between different samples (curve slope corresponds to the amount of cereulide in the sample). If the change of PMOT% over a time required for achieving PMOT% drop to 10% (ΔPMOT%/$\Delta\tau$) is plotted against the standard curve (obtained with known concentrations of valinomycin), a semi-quantitative estimation of the amount of cereulide in the sample is obtained. An optimized CASA method was applied to determine the production of cereulide under different conditions. No cereulide was found in aerated samples and in samples incubated at 12 °C. The amount of cereulide produced depended on the agar medium used, type of *Bacillus cereus* strain and the amount of oxygen present in the atmosphere.

Keywords: *Bacillus cereus*; Cereulide; Emetic toxin; Bioassay; Boar semen motility

1. Introduction

The lack of suitable, easy to handle cereulide detection method makes routine detection of this exotoxin in laboratory media and food samples difficult. A simple, yet powerful assay, based on the cereulide toxicity towards mitochondria was published (Andersson et al., 1998a,b, 2004; Haggblom et al., 2002). The assay is monitoring cease of boar sperm motility when exposed to cereulide. The toxin acts as an ionophor, transporting K^+ ions via the ion-carrier system into the mitochondria downstream of the electrical and concentration gradients. In this way it resembles the mode of action of valinomycin (Hoornstra et al., 2003). The damaged mitochondria fail in oxidoreductive functioning, causing changes in macroscopic behavior of semen cells, expressed as a decrease in cell motility (Andersson et al., 1998a,b, 2004). A possible observation of the semen motility can be done visually, as well as by means of the computer assisted sperm analysis

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doi:10.1016/j.mimet.2005.09.018

(CASA) systems. The main problems related to classical microscopic methods (i.e. subjectivity and variability) may result in nonreproducible results, especially when done on interlaboratory bases as it is dependent on the evaluator's skills. This in return results in high variations in the estimation of the motility parameters of same ejaculates assessed by different observers (Rijsselaere et al., 2003). Computerized measuring devices yield more objective results and allow for monitoring of multiple parameters simultaneously. One of the computer-aided semen analyzers, the Hamilton-Thorne, was previously validated for the evaluation of boar semen (Abaigar et al., 1999) and semen of some other mammalian species (Farrell et al., 1996). The main problems related to CASA application are the extreme needs for standardization of procedures, optimization of timing and validation of the system before any practical use is possible (Rijsselaere et al., 2003).

In the present study a CASA system (Hamilton Thorne Ceros 12.1) was used to determine the dynamics of boar semen motility changes under the toxic impact of cereulide on the semen mitochondria. In correlation with the effect of valinomycin on boar semen, a semi-quantitative system to detect cereulide concentrations in the range of 20–400 ng ml^{-1} was established. The detection system was further evaluated to determine the cereulide production by *Bacillus cereus* under various, food relevant, growth conditions.

2. Materials and methods

2.1. Optimization of the bioassay and of the semi-quantitative approach

2.1.1. Test protocol, material and apparatus

The boar semen motility test was applied with minor modifications to the originally described protocol (Andersson et al., 1998a,b, 2004). In short 195 μl of sperm was mixed with 5 μl of solution (blank or containing cereulide or valinomycin) in wells of a 37 °C pre-warmed microtiter plate (96 wells format) and immediately transferred into a Leja-slide (standard count 2-chamber 20 μm slide, Leja, Nieuw-Vennep, The Netherlands). Slides were pre-warmed on a portable stage warmer (automatic calibration to 37 °C). The HTR Ceros 12.1 (Hamilton Thorne Research, Beverly, USA) consists of a phase-contrast microscope (Olympus), a camera, a MiniTherm stage warmer, an image digitizer and a computer to save and analyze obtained data. If only a qualitative toxicity evaluation was required, undiluted samples were subjected to the test and results were, depending on concentration of cereulide present, obtained within 10–300 s. When a quantitative estimation was required the sample was serially diluted in two-fold dilution series requiring two consecutive dilution steps to give results within a semi-linear range of a valinomycin standard curve.

2.1.2. Boar semen

Boar semen (produced and provided by Hypor KI, Olsene, Belgium), either of the Belgian Piétrain extra muscled or the Beau-Pi boar, was on the production site diluted with extender containing sugar (to keep the spermatozoa alive), Na-EDTA as pH buffer, ions for the maintenance of osmotic balance and antibiotic (gentamycine) to control bacterial contamination. The sperm was standardized to a concentration of approximately 30 millions cells per milliliter with overall motility of more than 80% and packed in 100 ml plastic capped flasks. As recommended by Hypor KI the boar sperm was kept at temperatures between 15 °C and 20 °C (optimum 17 °C) and the fresh suspension was used for every day of analyses.

2.1.3. Effect of organic solvents and exposure time

The following organic solvents were used to dissolve and dilute cereulide: methanol (Fisher Scientific, Leicestershire, UK), ethanol (Vel, Leuven, Belgium) and dimethyl sulfoxide (DMSO, Sigma-Aldrich, Steinheim, Germany). All three solvents were tested in a ratio 40:1 (semen/solvent), as described above, in order to determine the one with the least influence on the boar spermatozoa motility. The contact time of solvent and semen is divided into two sequential periods. The period of the first 5 s, during which mixing of semen with solvent occurs and transfer of 7 μl of the mixture into the chamber of Leja slides, forms the pre-exposure time (pET). Leja slides are preset onto a heating-stage providing a uniform temperature of 37 °C on the slide. Since sperm motility and velocity are highly dependent on the temperature, assessment of the motility should be performed as close to 37 °C as possible (Iguer-ouada and Verstegen, 2001). At the moment of injection of the mixture into one of the Leja chambers, exposure time (ET) and observation of the semen behavior start. Motility changes are monitored on the computer screen by capturing the image and motility parameters every 5–10 s. The impact of the solvent on the percentage of semen progressive motility (PMOT%) is evaluated by calculating the average PMOT% and its standard deviation. The solvent inducing the smallest decrease in the PMOT% was chosen for further experiments.