



Establishment of a semi-automated pathogen DNA isolation from whole blood and comparison with commercially available kits

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ABSTRACT

Molecular methods for bacterial pathogen identification are gaining increased importance in routine clinical diagnostic laboratories. Achieving reliable results using DNA based technologies is strongly dependent on pre-analytical processes including isolation of target cells and their DNA of high quality and purity. In this study a fast and semi-automated method was established for bacterial DNA isolation from whole blood samples and compared to different commercially available kits: Looxster, MoLYsis kit, SeptiFast DNA isolation method and standard EasyMAG protocol. The newly established, semi-automated method utilises the EasyMAG device combined with pre-processing steps comprising human cell lysis, centrifugation and bacterial pellet resuspension. Quality of DNA was assessed by a universal PCR targeting the 16S rRNA gene and subsequent microarray hybridisation. The DNA extractions were amplified using two different PCR-mastermixes, to allow comparison of a commercial mastermix with a guaranteed bacterial DNA free PCR mastermix. The modified semi-automated EasyMAG protocol and the Looxster kit gave the most sensitive results. After hybridisation a detection limit of 10^1 to 10^2 bacterial cells per mL whole blood was achieved depending on the isolation method and microbial species lysed. Human DNA present in the isolated DNA suspension did not interfere with PCR and did not lead to non-specific hybridisation events.

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1. Introduction

The speed, specificity and sensitivity of pathogen identification are amongst the main advantages of applying molecular detection methods in comparison to conventional cultivation based identification assays (Wellinghausen et al., 2009; Jordan and Durso, 2000). Application of DNA-based technologies to pathogen identification allows accelerated time-to-result thereby fostering earlier initiation of antimicrobial therapy. Several molecular methods have been described for microbe identification, most of which rely on the detection of specific genomic sequences of the infectious pathogen. Amongst these techniques are polymerase chain reaction (PCR), real-time PCR, DNA microarrays and fluorescence in situ hybridisation (FISH). In order to achieve reliable and reproducible results, the applied target DNA must be of high purity and sufficient quantity (Mothershed and Whitney, 2006; Lehner et al., 2005; Shang et al., 2005; Wiesinger-Mayr et al., 2007; Yadav et al., 2005).

A sterile sampling method and an optimised sample preparation protocol are crucial to obtaining PCR compatible DNA targets (Radstrom et al., 2004). Complex biological samples such as blood represent a critical point for specific and sensitive identification of pathogens. Most biological samples contain substances that inhibit subsequent molecular methods (Al-Soud and Radstrom, 2001; Al-Soud et al., 2005). Plasma removal may eliminate interfering proteins, however other cellular blood components such as leukocytes contain high amounts of human DNA and other substances that are suspected to hamper subsequent molecular methods such as PCR. For this reason these cells must be eliminated either by lysis or mechanical removal. However, separation of blood components and blood cells still remains difficult because the success of various strategies is dependent on a number of different factors including plasma viscosity as well as the number and size of cells (Rock et al., 2000; Dzik et al., 2000).

Complete removal of inhibitors is difficult and time consuming. For this reason variations in the reaction mixture of subsequent techniques (e.g. PCR) are applied in an attempt to overcome these effects, e.g. changing enzymes and buffers (Wolfs et al., 2004). Also the addition of PCR enhancers has been shown to be very efficient (Al-Soud and Radstrom, 2000; Wilson, 1997).

In the past major emphasis has been placed on the improvement and acceleration of sample preparation for the application of

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molecular diagnostics. Consequently reports have been published recently which demonstrate improved approaches for DNA isolation from faeces, environmental or clinical samples (Nylund et al., 2010; Morin et al., 2010; de Boer et al., 2010). Additionally several ready to use kits have been developed for pathogen DNA extraction from clinical samples. Such systems include the MolYsis kit (MolzYM), the Looxster kit (SIRS-Lab), and the DNA isolation protocol of the SeptiFast kit (Roche diagnostics). However, these methods have to be carried out manually and are therefore difficult to implement in routine clinical diagnostic laboratories. On the other hand the EasyMAG is an automated DNA extraction device for human DNA from clinical samples. The recommended initial sample volume is 0.2 mL of whole blood to prevent a possible overload of the DNA binding silica beads with human DNA and other macromolecules. During an EasyMAG run DNA is purified and concentrated by binding to magnetic silica beads. In recent publications the applicability of magnetic silica beads for DNA separation was shown and further optimised. However, these applications still focus on human DNA starting with very small sample volumes (Duarte et al., 2010; Kang et al., 2009).

The human DNA depletion system of the MolYsis kit is based on selective lysis of blood cells using chaotropic buffers with subsequent degradation of released human DNA by chemically resistant DNases. Pathogens remain intact during this working step and are separated from the lysate by centrifugation. Resuspended microbes are then lysed by addition of muralytic enzymes (Disque et al., 2004, 2006). Released pathogen DNA is then purified and enriched using a purification column. The Looxster kit applies a complete cell lysis of whole blood by bead beating. After DNA isolation and purification the human and pathogen DNA are separated from each other. This is enabled by specific binding of pathogen DNA to a matrix contained in an enrichment column. The unbound eukaryotic DNA is eliminated during the subsequent washing steps. Bound bacterial DNA is then eluted and again purified by extraction. The product can then be used for further applications such as PCR. The SeptiFast kit lyses all cells by bead beating of whole blood. Isolated DNA is purified and harvested by application of a purification column. No separation of human and pathogen DNA is carried out.

In general the DNA suspension obtained and analysed in subsequent molecular techniques still contains a background of human DNA and further blood derived macromolecules, which can inhibit PCR. Furthermore the reagents of most suppliers are contaminated with bacterial DNA. This may lead to the generation of false positive results (Mühl et al., 2010; Handschur et al., 2009).

In this study we established and optimised a semi-automated isolation method for pathogen DNA from whole blood. It is based on using an automated EasyMAG device combined with the advantages of the MolYsis kit, which includes a human cell lysis step, human DNA depletion and a fast reduction of sample volume from 5 mL to 0.2 mL. The newly established method was compared to different commercially available kits developed for bacterial DNA isolation from whole blood. All protocols were evaluated with respect to DNA quantity and quality, through the application of sensitivity tests, comprising PCR amplification and subsequent microarray hybridisation. We further compared the amplification efficiency of a guaranteed DNA free PCR mastermix with a well established and sensitive mastermix, which was found to be contaminated with bacterial DNA.

2. Materials and methods

2.1. Strains and preparation of spiked blood

All experiments were carried out using clinical isolates which were obtained by routine culturing of microbes from blood cultures and subsequent commercial microbial identification techniques following the guidelines and procedures of CLSI (Clinical and Laboratory

Standards Institute). Pure cultures were obtained by cultivating the respective microbe in Caso bouillon overnight at 37 °C. Bacteria were harvested by centrifugation of 1 mL overnight cultures at 3000 g for 5 min. After discarding the supernatant, the bacterial pellet was washed in phosphate buffered saline (1x PBS) and harvested by centrifugation at 3000 g for 5 min. Microbe concentration per mL was finally adjusted using a McFarland 0.5 standard. Assays were performed using *Escherichia coli* (isolates 68307, 71122), *Staphylococcus aureus* (isolates 12998, 72171), *Klebsiella pneumoniae* (isolate 25809), *Enterococcus faecalis* (isolate 81239) and *Pseudomonas aeruginosa* (isolate 68961).

Fresh whole blood was obtained from the Austrian Red Cross and spiked to final concentrations of 10, 10² and 10³ bacteria per mL. The required volume for each method was then transferred to the prepared vials following the manufacturers' instructions.

2.2. DNA isolation

Bacterial DNA was isolated according to the manufacturers' instructions. The modified EasyMAG isolation included prelysis of blood cells and subsequent transfer of resuspended pellet to the device. Protocols for the different pathogen DNA isolation methods applied from whole blood are described in the following:

2.2.1. Modified EasyMAG DNA isolation

Before sample transfer to NucliSens lysis tubes the sample volume was decreased by lysis of human cells and harvesting bacterial pellet using MolYsis kit for 5 mL whole blood. 5 mL spiked whole blood were pipetted into a sterile 50 mL Falcon tube. 2 mL buffer CM was added, vortexed at full speed for 20 s and incubated at room temperature for 5 min. 2 mL buffer DB1 and 10 µL MolDNase B were added, vortexed for 20 s and incubated at room temperature for 15 min. Bacterial cells were harvested by centrifugation at 11,000 g for 10 min. The supernatant was discarded. The pellet was resuspended in 1 mL buffer RS and transferred to the NucliSens Lysis tubes. Further steps were carried out as described in the EasyMAG bacterial DNA isolation method:

2.2.2. EasyMAG bacterial DNA isolation (BioMerieux, Marcy l'Etoile, France)

NucliSens Lysis tubes for external cell lysis were removed from the refrigerator 15 min before use. 0.2 mL of spiked whole blood was added to the lysis tube and vortexed. Then 140 µL of magnetic silica (BioMerieux) was added to the lysis tube and incubated at room temperature for 10 min. The complete volume was transferred to the vial row and moved into the EasyMAG device. Sample number and volume were registered in EasyMAG software. Parameters such as probe volume (40 µL) and extraction programme (specific B extraction protocol 2.0.1) were set. Ready to use DNA was transferred from vial row to sterile 1.5 mL Eppendorf tubes.

2.2.3. MolYsis Complete (MolzYM, Bremen, Germany)

1 mL spiked fresh whole blood was transferred into a sterile 2 mL Eppendorf tube. 250 µL buffer CM was added and vortexed at full speed for 20 s. 250 µL buffer DB1 and 10 µL MolDNase B were added and vortexed immediately for 20 s. The suspension was incubated at room temperature for 15 min. Bacterial cells were harvested by centrifugation at 13,000 g for 10 min. The supernatant was decanted. Cells were washed by adding 1 mL buffer RS to bacteria pellet and vortexing for 10 s followed by centrifugation at 13,000 rpm for 5 min. The supernatant was discarded. 80 µL buffer RL was added and the pellet was resuspended by pipetting. 20 µL BugLysis solution was added and vortexed for 10 s followed by incubation in a thermomixer at 37 °C and 1000 rpm for 30 min. 150 µL buffer RP and 20 µL proteinase K were added and vortexed at full speed for 10 s followed by incubation in a thermomixer at 56 °C and 1000 rpm for 10 min.

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