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Comparison of commercially-available RNA extraction methods for effective bacterial RNA isolation from milk spiked samples

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Abstract Nucleic-acid based methods for bacterial identification are extremely useful in diagnostic applications due to their specificity and sensitivity. However, they require an optimal purification of the target molecules. As part of the development of a new diagnostic method for the detection of bacterial RNA in cow milk, we have compared four commercially available RNA extraction kits for the isolation of bacterial RNA from spiked UHT milk samples. The kits were compared in terms of extraction efficiency and RNA purity using two bacterial species, the Gram negative *Escherichia coli* and the Gram positive *Staphylococcus aureus*. Two kits are based in silica-matrix extraction, and the other two in the guanidinium thiocyanate-phenol-chloroform extraction. In our hands, the RNeasy Protect Bacteria Mini kit from QIAGEN was the best performing in terms of RNA yield, quality, reproducibility and consumable needs, under the conditions here described.

Keywords: bacteria, milk, purification, RNA

INTRODUCTION

Bacterial detection in cow milk can be an excellent tool in herd health management, lowering the costs associated to the control of herd diseases in dairy farms. Detection methods ideally have to be fast, sensible and species-specific, requirements all of them usually associated with high-throughput molecular detection techniques rather than traditional microbiological cultures. Current diagnostic methods are mainly based on microbiological cultures followed by biochemical tests on the isolated bacteria. Such methods are labor-intensive procedures that take at least 2 to 3 days to be completed (Cremonesi et al. 2006). Diagnostic methods based on nucleic acid detection are a promising alternative to current methods because they offer sensitivity, specificity and reduced time for bacterial identification (Forsman et al. 1997; Phuektes et al. 2003; Mothershed and Whitney, 2006). These methods are

usually based on the polymerase chain reaction (PCR) and they target specific DNA sequences, like the ribosomal RNA (rRNA) gene or the 16S-23S rRNA intergenic spacer of the ribosomal RNA operon (Forsman et al. 1997; Phuektes et al. 2003). No matter the technology used, bacterial concentrations in milk can be rather low and sensitivity thresholds below these concentrations are often a concern (Liu and Wilson, 2010). A nucleic-acid molecule present in high copy numbers per cell should thus be chosen as target to limit sensitivity problems. The 16S rRNA molecule is thought to be bearing most of the features desired in a target molecule. 16S rRNA in bacterial cells represents a large fraction of the total RNA present in the cell. Moreover, sequence conservation within the 16S rRNA is such that some regions are shared by all bacteria, some by species of the same genus, and some regions are species specific. Although RNA is more labile than DNA, there are currently different commercial kits that allow RNA isolation routinely in laboratory conditions. Nevertheless, to the best of our knowledge the efficiency of these kits has neither been tested, not compared, for the isolation of bacterial RNA from milk. Cow milk is a complex medium with constituents that could interfere with bacterial RNA isolation, for example, clogging silica columns used to trap nucleic acids or retaining bacterial cells into the fat fraction.

The purification of bacterial RNA requires first the disruption of the bacterial cells. In general, mechanical methods for cell disruption are not species specific but their efficiency is higher in comparison to any other method. These methods seem thus the best suited for their use in the dairy industry (Geciova et al. 2002). After cell lysis, further purification of the RNA from the cell debris has to be carried out in order to obtain a sample with the low presence of contaminants required for most molecular applications. Two main methods are used: a solid-phase extraction where RNA selectively binds to a matrix such as silica in the presence of high concentrations of chaotropic salts and the guanidinium-thyocianate phenol-cloroform extraction.

In this study we describe the comparison among four commercially available RNA extraction kits for the purification of bacterial RNA isolated from milk samples. Our goal was to identify the kit best suited to the needs of the new molecular methodologies for bacterial identification. More precisely, we wanted to assess their suitability for a new detection method targeting the 16S rRNA that we are developing for the dairy industry. We compared their efficiency in terms of RNA yield and purity using two bacterial species, *Escherichia coli* and *Staphylococcus aureus*, in culture broth and in milk spiked samples. We also analysed other features to take into account during the choice of the most suitable kit for dairy laboratories.

MATERIALS AND METHODS

Bacterial strains and growth conditions

Commercial strains of *Escherichia coli* CGSC 5073 and *Staphylococcus aureus* CECT240 were used in all experiments. Liquid and solid culture media were Luria Bertrani (LB) for *E. coli* and Nutrient Broth (NB) for *S. aureus*. Bacterial strains were maintained on agar plates at 4ºC. Pre-inoculum cultures (5 ml) were inoculated from a single colony pick and grown overnight. Growing conditions were 37ºC and 200 rpm. Cultures of 30 ml were inoculated with their respective pre-inoculums at an initial optical density at a wavelength of 550 nm (OD_{550}) of 0.05. OD_{550} was measured with Ultrospec 2100 Pro spectrophotometer using the respective culture media as blank.

The value of OD_{550} of 0.5 was used as the indicator for RNA extraction and the number of cells was verified by viable cell counting.

Viable cell counting

Viable cell counting was calculated at OD_{550} = 0.5 for *E. coli* and *S. aureus*. Growing curves and viable cell counting experiments were carried out in liquid and solid LB or NB media respectively. Culture media (100 ml) was inoculated with the overnight pre-inoculums at an initial OD_{550} of 0.05. One-ml samples were taken every hour to perform OD_{550} readings. OD_{550} was measured with Ultrospec 2100 pro spectrophotometer using 1 ml of the respective culture media as blank. For viable cell counting, 1 ml of culture at OD_{550} = 0.5 was serially diluted in duplicates in 0.9% NaCl solution. 100 µl of each dilution was plated in duplicate on LB or NB agar plates. Plates were incubated at 37ºC overnight. Plates with a number of colonies bellow 15 or over 300 were discarded. Viable cell counting was expressed as colony forming units per ml (cfu/ml).

RNA extraction: preparation of cell pellets

For RNA extraction, 1 ml of cultured cells at an OD_{550} = 0.5 were taken in triplicate. Samples were centrifuged at 6000 x g at room temperature (RT), supernatant was discarded and pellets were stored frozen at -20ºC until RNA extraction was performed. For all RNA extractions, frozen pellets were first resuspended in 300 µl of RNase free water (unless variations mentioned). For RNA extraction from milk, the 300 µl resuspended pellets were mixed with 1 ml of room temperature pre-warmed UHT milk, centrifuged again in the same conditions and the supernatant was discarded before performing RNA extraction. RNA extraction with RiboPure™-Bacteria Kit (Ambion) and the RNeasy Protect Bacteria Mini kit (Qiagen) was carried out following manufacturer's instructions. A mechanical disruption cycle was introduced in the NucliSENS® miniMAG® (Biomerieux) and TRIzol® Max Bacterial (Invitrogen) kits after cell pellet suspension in 300 µl of RNase free water. The resuspended cells were mixed with 2 ml NucliSENS® lysis buffer or TRIzol® reagent (kit provided), vortexed and further mechanically disrupted as in the QIAGEN and Ambion kits. The suspension was mixed with pre-weighted 0.1 mm glass beads (range 26-36 mg per ml of sample to disrupt) (Biospec Product Inc). Two cycles of beating of 2.5 min each, with one min on ice within cycles, were carried out in the MiniBead Beater (Biospec Product, Inc). Samples were centrifuged at 16000 x g for 1 min at RT and supernatant was transferred into a new tube. Nucleic-acid isolation was continued following manufacturer's protocol.

RNA yield and integrity

The RNA yield was evaluated in spectrophotometer (Ultrospec 2100 pro) using a precision cell quartz cubette of 10 mm light path and the OD at a wavelength of 260 nm (OD_{260}). The RNA was quantified as 1 OD_{260} corresponds to 40 µg ml^{-1} of RNA, and sterile water was used as blank. Obtained values were submitted to ANOVA analysis. RNA purity and integrity was analysed by electropherogram analysis using the Experion™ Automated Electrophoresis system (Bio-Rad) and the Experion RNA StdSens chips (5-500 ng/µl total RNA).

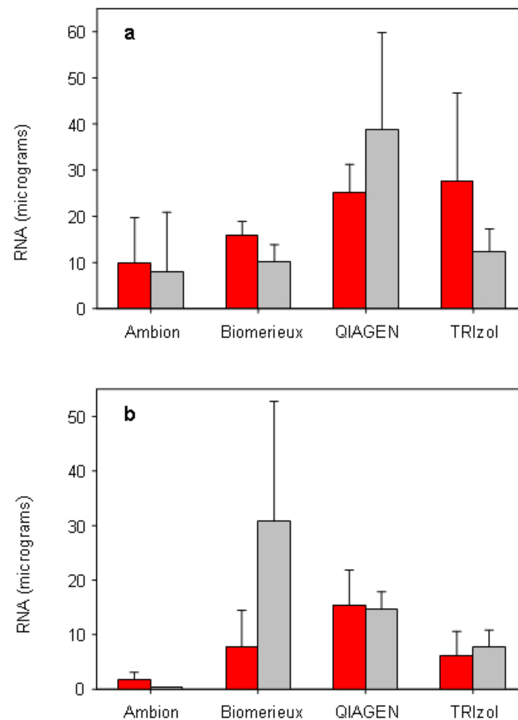

Fig. 1 Total RNA (µg) obtained from each commercial kit and each microorganism: *E. coli* (a) and *S. aureus* (b) from spiked-milk (grey bars) or culture media (red bars) samples.

RESULTS AND DISCUSSION

A fixed amount of cells for all RNA purification was determined in order to compare the different RNA extraction procedures. The different manufacturers' protocols recommended a concentration between 10^7-10^8 cfu/ml for optimal RNA extraction. We used the middle log phase point OD_{550} = 0.5 of *E. coli* as an indicator of exponential growth and we verified the same growing behaviour for *S. aureus*. We determined the amount of viable cells at OD_{550}=0.5 for both strains indicating a final correspondence of 1.4 x 10^8 and 2.8 x 10^8 cfu ml^{-1} for *E. coli* and *S. aureus* respectively.

In order to avoid culture variability, a culture of each strain was carried out up to a final OD_{550} of 0.5 and 1 ml centrifuged cell pellets were frozen by triplicate to use with each RNA extraction protocol. In order to achieve a comparable cell disruption, all kits were adapted to have a common bead beating step. Figure 1 shows the average values of RNA yield obtained with each of the kits. The QIAGEN kit gave the best RNA recovery values when testing *E. coli* samples and the kit was among the bests when using *S. aureus* ones (38.86 µg and 14.62 µg in spiked milk samples, respectively). Ambion kit yielded the worst results for both species in all studied cases (culture or spiked milk samples). The RNA recovery yield was higher for *E. coli* than

for *S. aureus* for approximately the same amount of cells in almost all cases. These results could be attributed to the thicker peptidoglycan network and the spherical coco shape of *S. aureus* that offers a higher rigidity and a higher resistence to disruption (Geciova et al. 2002; Huang et al. 2008; Vollmer et al. 2008). On the other hand, no significant differences were found by ANOVA analysis between obtained RNA yields from milk-spiked and milk-free extractions either for *E. coli* or *S. aureus* in all performed kits, although there was a tendency ($P = 0.0873$) with *S. aureus* and the Biomerieux kit for a greater RNA extraction from spiked milk samples compared to culture ones (30.75 µg versus 7.85 µg, respectively). These results suggest no major interference of fat, proteins and other milk components with the performance of the extraction processes tested in this study.

The quality of the isolated RNA samples was assessed with the Experion automated electrophoresis system (Bio-Rad). This method offers a higher sensitivity compared to other traditional methods such as the A 260:280 ratio, required specially to quantify samples with low RNA concentrations like the *S. aureus* samples. The Experion automated electrophoresis system employs Caliper Life Sciences' LabChip microfluidic separation technology and fluorescent sample detection to perform automated analysis of RNA samples. This apparatus produces electropherograms plotting relative fluorescence unit absorbed (vertical axis) and time (horizontal axis). Peaks that occur at a lower value indicate smaller nucleic acids that passed through the fluorescence emitter-sensor at an earlier time. Experion™ data output should be analyzed by visually inspecting the electropherogram: flat baseline punctuated with narrow peaks and a complete absence of broad, rounded peaks (Figure 2).

The highest RNA quality was identified by the absence of peaks other than the two peaks corresponding to the majority of cellular RNA, the 16S rRNA and the 23S rRNA. The analysis of the samples showed that the highest quality was obtained with the QIAGEN kit. Figure 2 shows the RNA profiles obtained from an *E. coli* sample extracted with the Biomerieux and the QIAGEN kits from culture media and milk-spiked samples. The Experion profiles of Figure 2 corroborate the highest RNA yields obtained with the QIAGEN kit and show a greater ratio of contaminants or RNA degraded forms in the Biomeriaux samples. Similar profiles were obtained with other samples indicating that QIAGEN performed the best extractions in terms of RNA quality. Moreover, the clear RNA profile was observed in all QIAGEN samples indicating good reproducibility.

Since the final goal of this study is the use of RNA as a target molecule for the identification of bacteria in milk, the most critical factor are the RNA yield as well as the RNA integrity which can be important in case of a bacterial detection based on large amplicons. Therefore these results suggested that the RNeasy Protect Bacteria Mini kit from QIAGEN is the method that best performed for this purpose. However for other applications requiring a precise quantification, an additional treatment with DNases in order to remove possible traces of genomic DNA would be needed.

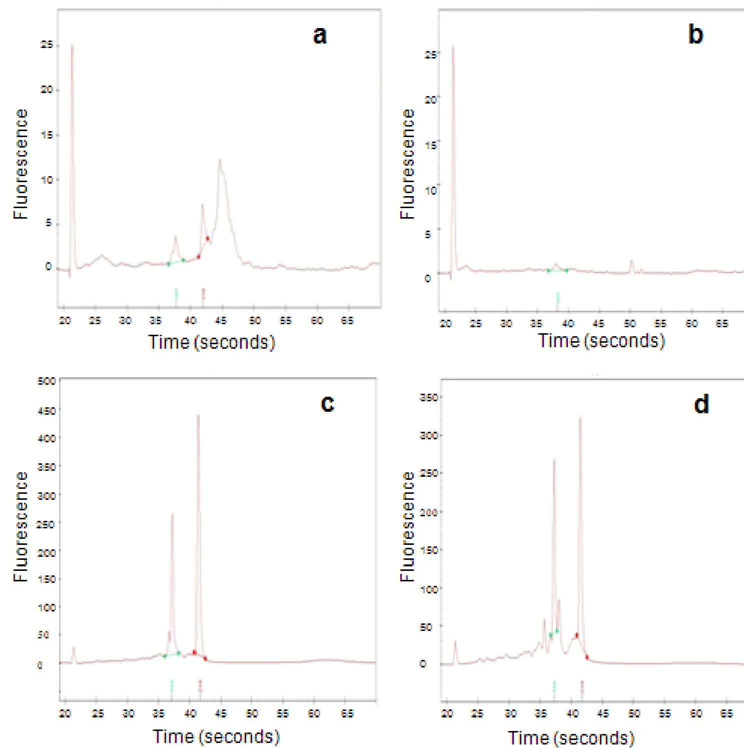

Fig. 2 Fluorescence profiles obtained using the Experion™ Automated Electrophoresis system (Bio-Rad) with total RNA samples isolated from *E. coli* culture (a, c) and *E. coli* spiked milk samples (b, d). RNA isolation was performed using either (a, b) the NucliSENS® miniMAG® kit (Biomerieux) or (c, d) the RNeasy Protect Bacteria Mini kit (QIAGEN). Dots underline fluorescence peaks corresponding to the ribosomal RNA.

The equipment cost and (user-friendliness) of the four commercial kits used in this study were also studied. The QIAGEN kit requires few and relatively cheap equipment, it is easy to carry out, it has no critical steps and it does not require hazardous agents (*i.e.* phenol) and it is potentially automatable, an important characteristic for high throughput techniques.

In conclusion, the RNeasy Protect Bacteria Mini kit from QIAGEN is the most appropriate commercial method for high bacterial RNA extraction in terms of RNA yield, quality, sample reproducibility and consumable needs, under the conditions described in this study.

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