Published in final edited form as:

J Microbiol Methods. 2007 February ; 68(2): 342–348.

Evaluation of microbial RNA extractions from *Streptococcus pneumoniae*

Ha-Sheng Li-Korotky^{a,b,*}, Lori A. Kelly^a, Otavio Piltcher^a, Patricia A. Hebda^{a,b}, and William J. Doyle^{a,b}

^a Division of Pediatric Otolaryngology, Children's Hospital of Pittsburgh, Pittsburgh, Pennsylvania, 15213, USA

^b Department of Otolaryngology, University of Pittsburgh School of Medicine, Pittsburgh, Pennsylvania, 15213, USA

Abstract

The mechanisms that control *Streptococcus pneumoniae*'s ability to colonize the nasopharynx or to invade the middle ear and cause acute otitis media are not understood. Focused study of these mechanisms requires efficient methods for the extraction of microbial RNA from minute clinical samples. Several lysis/extraction methods were tested and compared to determine the optimal conditions for isolating intact total RNA from pneumococcal cells. The sensitivity and efficiency of the extractions were evaluated by reverse transcription polymerase chain reaction (RT-PCR). Compared to other methods, mechanical homogenization in TRIZOL was the most efficient for releasing microbial RNA, and addition of polyinosinic acid (Poly I) as an RNA carrier increased the assay sensitivity to 10² colony forming units when detected by RT-PCR amplification of 16S ribosomal RNA or messenger RNA for penicillin binding protein 2b. Quantitative results were confirmed using a ribonuclease protection assay. Penicillin binding protein 2b was also detected in rat middle ear mucosa recovered 5 weeks after middle ear challenge with *S. pneumoniae*. This study describes a useful core methodology for use in identifying pneumococcal virulence genes from small titer samples and has promising applications in clinical studies of pneumococcal nasopharyngeal colonization and otitis media pathogenesis.

Keywords

Streptococcus pneumoniae; Microbial RNA extraction; Method assessment; Otitis media; Middle ear mucosa

1. Introduction

With the exception of colds, otitis media is the most common disease in infants and children, accounting for as many as 30 million pediatric office visits and annual expenditures of more than \$4 billion for diagnosis and treatment (Klein, 2001). *Streptococcus pneumoniae* (SP) is a major human pathogen, a leading cause of clinical acute otitis media (AOM) and responsible for over 1 million infant deaths each year from pneumonia, bacteremia and meningitis. With

*Corresponding author: Ha-Sheng Li-Korotky, Children's Hospital of Pittsburgh, Division of Pediatric Otolaryngology, 8140 Rangos Research Center, 3460 Fifth Avenue, Pittsburgh, PA 15213. Phone: (412) 692-6664; Fax: (412) 692-5075; Email address: Ha-Sheng.Li@chp.edu (H.S. Li-Korotky)..

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

the emergence and dissemination of antibiotic resistant pneumococcal strains and the limited SP subtype coverage for available vaccines, medical management has become increasingly complex and costly.

The host offers a variety of microenvironments for colonization/infection by a potential pathogen, and each, presents specific defense mechanisms that affect pathogen survival and reproduction. In response, pathogens have evolved permissive phenotypes that allow microenvironment invasion, and these are specified at the genomic level with or without conditional expression. For example, clinical SP isolates are highly heterogeneous with more than 90 pneumococcal serotypes having been identified based on unique and genome-restricted expression of specific cell-surface capsular polysaccharides (Claverys et al., 2000). More importantly, SP (and other bacteria) presents conditional phenotypes that reflect transcriptional regulation of gene complexes within the SP genome that promote the organisms survival under changing microenvironments (Dowson et al., 1997; Hakenbeck et al., 2001). One manifestation of this is the intra-colony change in morphology, visualized as differences in opacity, and referred to as phase variants (Saluja et al., 1995; Weiser et al., 1994; Weiser, 1998). Phase variant-dependent, *in vivo* virulence differences were demonstrated for SP in an infant rat model of nasopharyngeal colonization (Weiser et al., 1994), in a murine model of sepsis (Kim et al., 1998; Kim et al., 1999), and in chinchilla and rat models of AOM (Long et al., 2003; Tong et al., 2001).

In vivo (infected blood and cerebrospinal fluid) and *in vitro* (pharyngeal epithelial cell line) global SP gene expression was assayed using whole-genome microarrays for exposures to a variety of extant environmental conditions (Orihuela et al., 2004b). Those results showed that: SP virulence gene expression is much greater for the *in vivo* when compared to *in vitro* protocols; is tissue specific (Orihuela et al., 2004a; Orihuela et al., 2004b), and is regulated at the levels of mRNA transcription and/or stability (Martin-Galiano et al., 2004). In passage-attenuated, poorly infective SP strains, significant downregulation of the previously identified virulence factors, *psaB*, *psaC* and *psaA*, was demonstrated (Mccluskey et al., 2004).

By extension, it is expected that application of these techniques and methods to SP colonization of the nasopharynx and secondary infection of the middle ear will provide a deeper understanding of AOM pathogenesis, which in turn, will provide leads regarding targeted intervention. Such applications require that bacteria be recovered from the compartments of interest (nasopharynx, middle ear) where extant colony representation may be low, that high-quality mRNA be extracted from the bacterial species of interest, and that the mRNA be assayed quantitatively using highly sensitive and specific methods. This report focuses on the second and third steps in this process and compares several extraction methods commonly used to isolate mRNA when coupled to different RNA detection methods for maximal yield of high quality mRNA from the lowest SP starting titer. The optimal method was then applied to middle ear mucosa (MEM) recovered from rats that had been previously challenged with SP.

2. Materials and methods

2.1. Reagents

TRIZOL, vanadyl ribonucleoside complex (VRC), and reagents for reverse transcription were purchased from GIBCO BRL (Gaithersburg, MD, USA) and all other reagents were purchased from Sigma Chemical Co (St. Louis, MO, USA).

2.2. Design

2.2.1. Study rationale—Bacterial RNA has different levels of intracellular stability, with the tRNA and rRNA representing stable species and mRNA being inherently unstable with a

half-life ranging from 0.5 to 20 minutes (Bernstein et al., 2002; Bernstein et al., 2004; Carpousis et al., 1999; Hambræus et al., 2003; Patel et al., 1991). While mRNA in bacteria is usually degraded from the 3' polyadenylated termini (Kushner, 2004), the lack of polyadenylated transcripts excludes enrichment of mRNA by affinity purification. Consequently, the primary endpoint for determining extraction efficiency was total bacterial RNA. For mRNA quantification, an oligo(dT)₁₂₋₁₈ primer was used for reverse mRNA transcription to enrich the cDNAs that are generated from bacterial polyadenylated RNA (mRNA).

2.2.2. Study protocol—The study protocol consisted of a series of *in vitro* experiments designed to evaluate the efficiency of different extraction techniques for isolation of total SP RNA. The experimental conditions were 1) sonication in TRIZOL, 2) homogenization in TRIZOL, 3) mutanolysin treatment, 4) mutanolysin with proteinase K and sodium dodecyl sulfate (SDS), 5) mutanolysin with homogenization in TRIZOL, and 6) Polyinosinic acid (Poly I) with homogenization in TRIZOL (Table). The technique of homogenization in TRIZOL was also applied to a pooled rat MEM specimen recovered 5 weeks after middle challenge with SP.

2.3. Bacterium

SP type 6AP2 was obtained from the Center for Disease Control and underwent animal (rats) passage twice prior to use. The culture conditions for achieving maximal growth and the growth curves for those conditions were defined. Bacteria for the experiments were harvested and frozen just prior to the termination of exponential growth so as to collect highly viable bacterial cells. Recovered bacteria were concentrated to 9.4×10^8 CFU/mL and stored in Tryptic Soy Broth Buffer (TSB, pH 7.3) (Difco Laboratories, Detroit, MI, USA) at -80°C until use. Bacterial serial dilutions (total volume 50 μL) were made in 1M Potassium Phosphate Buffer (PPB, pH 8.0) to log concentrations (10^2 to 10^7 CFU). The ABS_{600} of a bacterial dilution containing 1×10^7 CFU/50 μL was 0.210.

2.4. Release of bacterial RNA

2.4.1. Sonication in TRIZOL—TRIZOL (1:10) was added to the samples which were then sonicated at 0°C for 1 h, and stored at -80°C until extraction.

2.4.2. Homogenization in TRIZOL—TRIZOL was added to each sample (1:10) and the mix was homogenized using a variable speed drill (setting 1–3 for 30 seconds) attached to a disposable, plastic pestle that was mounted within a specially “etched” homogenization tube. Processed samples were stored at -80°C until extraction.

2.4.3. Mutanolysin treatment—This cell lysis procedure is used as a first step for isolation of nucleic acids and intact rRNA from Gram-positive bacteria (Fliss et al., 1991). Samples, were centrifuged at $12,500 \times g$ for 10 min at 4°C , cell pellets washed with 500 μL of 1M PPB (pH 8.0), suspended in 500 μL of ice cold acetone and kept on ice for 10 min. After centrifugation, the acetone was decanted and the cells were suspended in 200 μL of lysis buffer containing 30 U of mutanolysin, 100 mM PPB (pH 6.2), and 10 mM VRC. The final mixture was incubated for 30 min at 37°C and centrifuged at $12,500 \times g$ for 15 min to remove cell debris. The supernatant was recovered and stored at -80°C until extraction.

2.4.4. Mutanolysin with proteinase K and SDS—The samples were treated as described for the mutanolysin protocol except that after the 30 min incubation at 37°C , the samples were treated with 10 μg of proteinase K and 0.005% SDS. This protocol was previously used to obtain *Streptococcus thermophilus* purified cell walls (Quiberoni et al., 2000). Samples were then incubated at 50°C for 30 min, at 70°C for 10 min and centrifuged at $12,500 \times g$ for 15 min to remove cell debris. The supernatant was recovered and stored at -80°C until extraction.

2.4.5. Mutanolysin with homogenization in TRIZOL—The samples were treated as described in the mutanolysin protocol, except that after the 30 min incubation at 37°C, 500 µL of TRIZOL was added to each sample. All samples were homogenized with maximum speed for 30 sec using custom-designed plastic tubes and disposable pestles. The samples were stored at – 80°C until extraction.

2.4.6. Poly I with homogenization in TRIZOL—A previously published protocol used for extracting *E. coli* RNA was followed (Winslow et al., 1991). Polyinosinic acid (Poly I) (30 µg) was added to each bacterial dilution to act as an RNA carrier. TRIZOL reagent was added to bacteria/Poly I mix that was then homogenized at the maximum speed for 30 sec and stored at – 80°C until extraction.

2.5. In Vivo samples and release of cellular RNA

The experimental protocol was reviewed and approved by the Animal Research and Care Committee at the Children's Hospital of Pittsburgh (Animal Welfare Assurance No. A3617-01). The animal model and tissue collection were reported before (Li et al., 2002). Rat MEM was collected from two rats at 5 weeks after middle ears were challenged with 2.25×10^6 CFUs of SP. Specimens underwent mechanical homogenization in TRIZOL, as described in the Section 2.4.2., with subsequent RNA extraction.

2.6. RNA extraction and DNase I treatment

RNA extraction was performed by following the instructions of TRIZOL manufacturer. All samples previously kept at –80°C were thawed on ice followed by 5 min at room temperature. Chloroform (200 µL) was added to each sample and the mix was vortexed for 1 minute and kept at room temperature for 3 min. After centrifugation at $12,000 \times g$ for 15 min at 4°C, the clear supernatant (containing the RNA) was saved on ice and 10 µg of glycogen was added to each sample. The RNA precipitation was carried out by adding 500 µL of isopropanol to each sample, incubation at room temperature for 30 min and centrifugation at $12,000 \times g$ for 15 min. The RNA pellets were washed with 1 mL of 75% ethanol in DEPC-treated water, followed by centrifugation at $12,000 \times g$ for 10 min. The pellets were dried and suspended in an appropriate volume of DEPC-water according to the size of the pellet (10 ~ 50 µL). All RNA samples were subjected to treatment with DNase I (200 units) (GenHunter Co., Nashville, TN, USA) at 37°C for 30 min, followed by phenol/chloroform extraction. For the samples containing VRC, a 10-fold molar excess of EDTA over the VRC was added just prior to the addition of ethanol to prevent the co-precipitation of RNA with VRC.

2.7. Evaluation of RNA quality and quantity

To evaluate the efficiency and sensitivity of a particular lysis/extraction method, RNA quality and quantity were assessed using four methods: 1) 1.5% agarose/Tris-Borate-EDTA (TBE) gel electrophoresis and ethidium bromide staining, 2) assessment of 260/280 absorbance (ABS) ratio in a Beckman DU 600 Spectrophotometer (Beckman, Fullerton, CA, USA), 3) RT-PCR amplification; and 4) Ribonuclease protection assay (RPA).

2.7.1. Reverse transcription and polymerase chain reaction (RT-PCR)—For the reverse transcription reaction (in duplicate), a mixture of total SP RNA (0.3 µg) and an oligo (dT)_{12–18} primer (0.5 µg/µL) (GIBCO Life Technologies, Gaithersburg, MD) was incubated at 75°C for 10 min followed at 42°C for 2 min. A reaction buffer containing 1X reaction buffer (250 mM Tris-HCl pH 8.3, 375 mM KCl and 15 mM MgCl₂), 10 mM DTT and 1mM dNTPs was then added. After incubation at 42°C for 2 min, SuperScript II (200 units) (GIBCO) was added. This mixture was then incubated at 42°C for 58 min followed by 70°C for 15 min in a 9600 thermocycler (Applied Biosystems, Foster City, CA, USA). Gene specific primers for

the amplification of SP cDNA were selected based on SP genomic sequence and a previous publication. For 16S rRNA, up-stream (5'-GGC TAC ACA CGT GCT ACA ATG G-3') and down-stream (5'-CGA CTT CGG GTG TAC AAA CTC TC-3') primers were designed. For penicillin binding protein 2b (pbp2b), up-stream (5'-ATG CAG TTG GCT CAG TAT GTA-3') and down-stream (5'-CAC CCA GTC CTC CCT TAT CA-3') primers were adapted from a previous publication (Zhang et al., 1995). All primers were synthesized by GIBCO BRL. A 25- μ L PCR reaction included 2 μ L of cDNA, gene-specific primers (0.4 μ M), 0.4 mM dNTPs, and AmpliTaq DNA polymerase (Applied Biosystems). PCR was performed for 35 cycles with temperature at 94°C for 15 seconds, at 55°C for 1 min, and at 72°C for 1 min on a thermocycler 9600 (Applied Biosystems). PCR products were size-fragmented on a 2.5% agarose/Tris-Acetate-EDTA gel.

2.7.2. Ribonuclease protection assay (RPA)—PCR products containing 16S rRNA cDNA were recovered and purified using a Wizard PCR Preps DNA Purification System (Promega, Madison, WI, USA). The purified PCR product was cloned using pGEM-T Vector System II (Promega). The cloned 16S rRNA was purified using the QIAGEN Maxi Kit (Qiagen, Valencia, CA, USA), sequenced using T7 and SP6 primers and reagents supplied in an ABI Prism BigDye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems), and subsequently linearized using Spe I (T7) and Sac II (SP6) restriction enzymes. *In vitro* transcription of the 16S linearized plasmid DNA was performed using Ambion's MAXIScript Kit (Ambion, Inc., Austin, TX, USA). Reactions were set up using 50 μ Ci of p32-UTP (ICN Biomedicals, Inc., Costa Mesa, CA, USA) per reaction according to manufacturer's instructions. The radiolabeled RNA transcripts were gel purified and quantified by scintillation counting. RNA was extracted from decreasing concentrations of SP by mechanical homogenization in TRIZOL/Poly I. These samples were hybridized with 5×10^4 cpm/reaction of each 16S rRNA according to manufacturer's instructions. The hybridized products were run on a 5% polyacrylamide TBE-Urea gel (BIO-RAD) and exposed to X-Ray film for 16 hrs.

3. Results

Homogenization in TRIZOL resulted in consistent recovery of SP RNA from high titer samples (10^6 CFU). In contrast, neither sonication of the test samples in TRIZOL nor sample pretreatment with mutanolysin followed by mechanical homogenization in TRIZOL produced visible RNA bands on ethidium bromide stained agarose gels (Table). SP samples treated with either mutanolysin alone or mutanolysin/proteinase K/SDS without homogenization in TRIZOL resulted in detection of degraded bacterial RNA only at very high SP titers (10^7 CFU), suggesting that this processing causes significant RNA degradation (Fig. 1).

Figure 2 shows respectively the mammalian and bacterial RNA bands on ethidium bromide stained agarose gels for rat MEM samples and SP test samples when extracted by homogenization in TRIZOL. The distinct bands for the 28S/18S rRNA (for MEM) and the 16S/23S rRNA (for *in vitro* bacterial samples) show that this method is efficient for isolating RNA. However, while the mammalian RNA was detected at relatively high abundances, detection of the bacterial RNA from the SP test samples was limited to those with high bacterial titers.

Detection of bacterial RNA in the SP test samples was enhanced by adding polyinosinic acid (Poly I) as an RNA carrier and using RT-PCR amplification of transcripts coding for the 16S rRNA and the pbp2b mRNA (Figs. 3A and 3B). While Poly I masked the RNA OD_{260/280} reading and blocked RNA visualization on agarose gels for sample titers less than 10^6 CFU (Fig. 2), it increased the RT-PCR sensitivity by five orders of magnitude to SP detection levels of approximately 10^2 CFU. Of interest, pbp2b gene expression was also detected in rat MEM samples collected 5 weeks after middle ear challenge with SP when the homogenization in

TRIZOL method was used (Fig. 4). The efficiency and sensitivity of the TRIZOL/Poly I method was confirmed using RPA and that assay demonstrated SP 16S rRNA expression to be proportional to input SP titer (Fig. 5).

4. Discussion

As with other bacteria, SP, the major bacterial cause of AOM, responds to changes in the local environment by selective regulation of mRNA gene transcripts in patterns that favor survival under the revised extant conditions. For example, one study reported that transcription of approximately 38% (658 genes) of the SP genome (1717 genes) was modified (1.5-fold) by varying temperature over a range from 21 to 40 °C (Pandya et al., 2005). Moreover, this gene expression cycling is extremely rapid with the phase delay between environmental signal and transcript expression measured in minutes and the subsequent silencing of gene expression being under constitutive control by processes that rapidly degrade “free” mRNA (Bernstein et al., 2002; Bernstein et al., 2004; Hambraeus et al., 2003; Patel et al., 1991). The phenotypic expressions of these transcriptional/translational responses include assayable protein products some of which are functionally recognized as “virulence factors”. Recent studies emphasize the important role played by “virulence factor” expression in controlling host-pathogen interactions and show that modulating factor expression is prerequisite for transformations between colonization and bacterial infection with/without tissue invasion (Weiser, 1998).

This developing paradigm of evolving host-pathogen interactions holds promise for understanding the pathological role played by SP in causing AOM and for developing rational approaches to prevent a high frequency of all AOM episodes by intervening in that process. As a prelude to formal studies of AOM, it is necessary to establish reliable and sensitive methodologies for: recovery of SP from potentially low titer regions (e.g. middle ear, nasopharynx); high yield isolation of the target RNA species (bacterial/host mRNA, rRNA), and quantification of specific transcripts with known protein products related to virulence. This goal is complicated by the aforementioned rapid expression cycling at the mRNA level and the consequent need for a time-limited processing protocol.

In this study, six RNA extraction protocols were compared for their quantitative efficiency to extract total SP RNA from samples varying over a 6 log range of titers. The coupled efficiency of two methods for RNA quantification, PCR and spectrophotometry, was also determined. Physical cell wall disintegration by sonication was previously described for RNA isolation in organisms with difficult-to-lyse cell walls (Frostegard et al., 1999; Rantakokko-Jalava et al., 2002), but in the present study, this method was not effective for isolating SP RNA. Enzymatic digestion by mutanolysin treatment or mutanolysin combined with proteinase K and SDS was also reported to yield high quality RNA and DNA from gram positive bacteria (Ampe et al., 1998; Fliss et al., 1991; Gopalakrishna et al., 1981; Shiroza et al., 1998; Simpson et al., 1993; Zhou et al., 1996), but again, the present study documented only a low RNA yield for these procedures and suggested that the required sample incubations at 50 °C ~ 37 °C may have been responsible by causing significant RNA degradation. In contrast, homogenization of SP in TRIZOL did release bacterial RNA and when modified as mechanical homogenization of samples containing polyinosinic acid (Poly I) in TRIZOL and combined with PCR detection of product yielded RNA quantification proportional to the SP titer of the sample. Moreover, the method of the mechanical homogenization in TRIZOL was shown to detect bacterial mRNA (pbp2b) in a pooled rat MEM sample recovered 5 weeks after middle ear challenge with SP, though the significance of this single observation to AOM pathogenesis must await the results of future experiments with a specific focus on that issue.

The high sensitivity of this protocol for extraction/detection of SP RNA depends on the addition of Poly I to the reaction mixture. There, Poly I may act to bind bacteria by complexing with

lipopolysaccharide of Gram-negative and lipoteichoic acid of Gram-positive bacteria (Peiser et al., 2001). An important control observation for the interpretation of the sensitivity data is provided by the results of a recent study that showed Poly I alone did not prime detectable cDNA synthesis nor did it inhibit cDNA synthesis when primed by cellular mRNA (Winslow et al., 1991). However, in tissues, Poly I was associated with the local production of inflammatory mediators (Campa et al., 2005). Therefore, when applying this method of bacterial RNA extraction/detection to tissue samples, it may be necessary to first deactivate tissue samples in the TRIZOL solution before adding the Poly I. Current experiments are in progress to explore this possibility.

In summary, homogenization of fluid and MEM samples with Poly I in TRIZOL, when combined with PCR detection of RNA product, is a highly efficient method for quantitative detection of SP (and host) RNA. As such, the method represents a promising core technology for the experimental and clinical study of the evolving host-pathogen interactions involved in AOM pathogenesis. Applications focused on describing the diachronic changes in SP transcriptome expression for transition times between nasopharyngeal colonization and acute middle ear infection are currently planned for testing in animal models of SP AOM.

Acknowledgements

This work was supported by Public Health Service grants DC007511 (HSLK) and DC001260 (WJD and PAH) from the National Institute on Deafness and Other Communication Disorders, and funds made available from the Lester A. Hamburg Endowed Fellowship in Pediatric Otolaryngology and the Eberly Family Endowed Chair in Pediatric Otolaryngology (Dr. Charles D. Bluestone, Endowed Chair in Pediatric Otolaryngology, Children's Hospital of Pittsburgh). We thank Ms. Chia-Yee Lo and Ms. Julianne M. Banks for technical assistance.

References

- Ampe F, Ben Omar N, Guyot JP. Recovery of total microbial RNA from lactic acid fermented foods with a high starch content. *Lett Appl Microbiol* 1998;27:270–274. [PubMed: 9830143]
- Bernstein JA, Khodursky AB, Lin PH, Lin-Chao S, Cohen SN. Global analysis of mRNA decay and abundance in *Escherichia coli* at single-gene resolution using two-color fluorescent DNA microarrays. *Proc Natl Acad Sci USA* 2002;99:9697–9702. [PubMed: 12119387]
- Bernstein JA, Lin PH, Cohen SN, Lin-Chao S. Global analysis of *Escherichia coli* RNA degradosome function using DNA microarrays. *Proc Natl Acad Sci USA* 2004;101:2758–2763. [PubMed: 14981237]
- Campa VM, Iglesias JM, Carcedo MT, Rodriguez R, Riera J, Ramos S, Lazo PS. Polyinosinic acid induces TNF and NO production as well as NF-kappaB and AP-1 transcriptional activation in the monocytemacrophage cell line RAW 264.7. *Inflamm Res* 2005;54:328–337. [PubMed: 16158333]
- Carpousis AJ, Vanzo NF, Raynal LC. mRNA degradation. A tale of poly(A) and multiprotein machines. *Trend Genet* 1999;15:24–28.
- Claverys JP, Prudhomme M, Mortier-Barriere I, Martin B. Adaptation to the environment: *Streptococcus pneumoniae*, a paradigm for recombination-mediated genetic plasticity? *Mol Microbiol* 2000;35:251–259. [PubMed: 10652087]
- Dowson CG, Barcus V, King S, Pickerill P, Whatmore A, Yeo M. Horizontal gene transfer and the evolution of resistance and virulence determinants in *Streptococcus*. *Soc Appl Bacteriol Symp Ser* 1997;26
- Fliss I, Emond E, Simard RE, Pandian S. A rapid and efficient method of lysis of *Listeria* and other gram-positive bacteria using mutanolysin. *Biotechniques* 1991;11:456–457.
- Frostegard A, Courtois S, Ramisse V, Clerc S, Bernillon D, Le Gall F, Jeannin P, Nesme X, Simonet P. Quantification of bias related to the extraction of DNA directly from soils. *Appl Environ Microbiol* 1999;65:5409–5420. [PubMed: 10583997]
- Gopalakrishna Y, Langley D, Sarkar N. Detection of high levels of polyadenylate-containing RNA in bacteria by the use of a single-step RNA isolation procedure. *Nucleic Acids Res* 1981;9:3545–3554. [PubMed: 6169008]

- Hakenbeck R, Balmelle N, Weber B, Gardes C, Keck W, De Saizieu A. Mosaic genes and mosaic chromosomes: intra- and interspecies genomic variation of *Streptococcus pneumoniae*. *Infect Immun* 2001;69:2477–2486. [PubMed: 11254610]
- Hambraeus G, Von Wachenfeldt C, Hederstedt L. Genome-wide survey of mRNA half-lives in *Bacillus subtilis* identifies extremely stable mRNAs. *Mol Genet Genomics* 2003;269:706–714. [PubMed: 12884008]
- Kim JO, Romero-Steiner S, Sorensen UB, Blom J, Carvalho M, Barnard S, Carlone G, Weiser JN. Relationship between cell surface carbohydrates and intrastain variation on opsonophagocytosis of *Streptococcus pneumoniae*. *Infect Immun* 1999;67:2327–2333. [PubMed: 10225891]
- Kim JO, Weiser JN. Association of intrastain phase variation in quantity of capsular polysaccharide and teichoic acid with the virulence of *Streptococcus pneumoniae*. *J Infect Dis* 1998;177:368–377. [PubMed: 9466523]
- Klein JO. The burden of otitis media. *Vaccine* 2001;19:S2–8. [PubMed: 11163456]
- Kushner SR. mRNA decay in prokaryotes and eukaryotes: different approaches to a similar problem. *IUBMB Life* 2004;56:585–594. [PubMed: 15814456]
- Li HS, Doyle WJ, Swarts JD, Hebda PA. Suppression of epithelial ion transport transcripts during pneumococcal acute otitis media in the rat. *Acta Oto-Laryngol (Stockholm)* 2002;122:488–494.
- Long JP, Tong HH, Shannon PA, Demaria TF. Differential expression of cytokine genes and inducible nitric oxide synthase induced by opacity phenotype variants of *Streptococcus pneumoniae* during acute otitis media in the rat. *Infect Immun* 2003;71:5531–5540. [PubMed: 14500471]
- Martin-Galiano AJ, Wells JM, De La Campa AG. Relationship between codon biased genes, microarray expression values and physiological characteristics of *Streptococcus pneumoniae*. *Microbiology* 2004;150:2313–2325. [PubMed: 15256573]
- Mccluskey J, Hinds J, Husain S, Witney A, Mitchell TJ. A two-component system that controls the expression of pneumococcal surface antigen A (PsaA) and regulates virulence and resistance to oxidative stress in *Streptococcus pneumoniae*. *Mol Microbiol* 2004;51:1661–1675. [PubMed: 15009893]
- Orihuela CJ, Gao G, Francis KP, Yu J, Tuomanen EI. Tissue-specific contributions of pneumococcal virulence factors to pathogenesis. *J Infect Dis* 2004a;190:1661–1669. [PubMed: 15478073]
- Orihuela CJ, Radin JN, Sublett JE, Gao G, Kaushal D, Tuomanen EI. Microarray analysis of pneumococcal gene expression during invasive disease. *Infect Immun* 2004b;72:5582–5596. [PubMed: 15385455]
- Pandya U, Allen CA, Watson DA, Niesel DW. Global profiling of *Streptococcus pneumoniae* gene expression at different growth temperatures. *Gene* 2005;360:45–54. [PubMed: 16154298]
- Patel BK, Banerjee DK, Butcher PD. Characterization of the heat shock response in *Mycobacterium bovis* BCG. *J Bacteriol* 1991;173:7982–7987.
- Peiser L, Gordon S. The function of scavenger receptors expressed by macrophages and their role in the regulation of inflammation. *Microb Infect* 2001;3:149–159.
- Quiberoni A, Stiefel JJ, Reinheimer JA. Characterization of phage receptors in *Streptococcus thermophilus* using purified cell walls obtained by a simple protocol. *J Appl Microbiol* 2000;89:1059–1065. [PubMed: 11123479]
- Rantakokko-Jalava K, Jalava J. Optimal DNA isolation method for detection of bacteria in clinical specimens by broad-range PCR. *J Clin Microbiol* 2002;40:4211–4217. [PubMed: 12409400]
- Saluja SK, Weiser JN. The genetic basis of colony opacity in *Streptococcus pneumoniae*: evidence for the effect of box elements on the frequency of phenotypic variation. *Mol Microbiol* 1995;16:215–227. [PubMed: 7565084]
- Shiroza T, Shinozaki N, Watanabe T, Ikemi T, Fukushima K, Abiko Y. Rapid isolation of chromosomal DNA from oral streptococci and polymerase chain reaction-oriented restriction fragment-length polymorphism analysis for genetic heterogeneity. *Oral Microbiol Immun* 1998;13:11–16.
- Simpson CL, Giffard PM, Jacques NA. A method for the isolation of RNA from *Streptococcus salivarius* and its application to the transcriptional analysis of the *gtfJK* locus. *FEMS Microbiol Lett* 1993;108:93–97. [PubMed: 7682529]

- Tong HH, Weiser JN, James MA, Demaria TF. Effect of influenza A virus infection on nasopharyngeal colonization and otitis media induced by transparent or opaque phenotype variants of *Streptococcus pneumoniae* in the chinchilla model. *Infect Immun* 2001;69:602–606. [PubMed: 11119562]
- Weiser JN. Phase variation in colony opacity by *Streptococcus pneumoniae*. *Microb Drug Resist* 1998;4:129–135. [PubMed: 9651000]
- Weiser JN, Austrian R, Sreenivasan PK, Masure HR. Phase variation in pneumococcal opacity: relationship between colonial morphology and nasopharyngeal colonization. *Infect Immun* 1994;62:2582–2589. [PubMed: 8188381]
- Winslow SG, Henkart PA. Polyinosinic acid as a carrier in the microscale purification of total RNA. *Nucleic Acids Res* 1991;19:3251–3253. [PubMed: 1712097]
- Zhang Y, Isaacman DJ, Wadowsky RM, Rydquist-White J, Post JC, Ehrlich GD. Detection of *Streptococcus pneumoniae* in whole blood by PCR. *J Clin Microbiol* 1995;33:596–601. [PubMed: 7751363]
- Zhou J, Bruns MA, Tiedje JM. DNA recovery from soils of diverse composition. *Appl Environ Microbiol* 1996;62:316–322. [PubMed: 8593035]

Fig. 1. Degraded bacterial RNA on 1.5% agarose/TBE/EtBr gels from *S. pneumoniae in vitro* (10^{5-7} CFUs, in duplicate) specimens extracted using mutanolysin or mutanolysin plus proteinase K/SDS, respectively.

Fig. 2. Mammalian and bacterial RNA patterns (1.5% agarose/TBE/EtBr gel) for rat middle ear mucosa (MEM, in duplicate) and *in vitro* *S. pneumoniae* (10⁵⁻⁷ CFUs, in duplicate) samples extracted using the TRIZOL or TRIZOL/Poly I method. L1: RNA marker. L2: DNA marker.

Fig. 3. Gene expression of 16S rRNA (218 bp) (A) and penicillin binding protein 2b (pbp2) (106 bp) (B) as assayed using PCR (2% agarose/TAE gel) for *in vitro* *S. pneumoniae* (10²⁻⁷ CFUs, in duplicate) samples extracted using mechanical homogenization in TRIZOL/Poly I (right panel) and mechanical homogenization in TRIZOL (left panel). L: DNA marker.

Fig. 4. Penicillin binding protein 2b (pbp2) gene expression (106 bp) in bacterial infected middle ear mucosa (BM, in duplicate) recovered 5 weeks after pneumococcal challenge, non-infected middle ear mucosa (NM), and normal rat pancreas (P). RNA was extracted using the mechanical homogenization in TRIZOL and assayed by RT-PCR (2% agarose/TAE gel). NC, negative control (without cDNA); L, DNA marker.

Fig. 5. Visual banding results (2% agarose/TAE gel) for the RNase protection assay showing that signal intensity of 16S rRNA decreased with decreasing log *S. pneumoniae* titer. NC, negative control.

Table

Comparative results for SP RNA detection by RT-PCR after application of each of six lysis/extraction methods

Method	Sensitivity
Sonication in TRIZOL	-
Mechanical homogenization in TRIZOL	10^6 CFUs
Mutanolysin treatment	10^7 CFUs
Mutanolysin/proteinase K/SDS	10^7 CFUs
Mutanolysin/mechanical homogenization in TRIZOL	-
Poly I/mechanical homogenization in TRIZOL	10^2 CFUs