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Original article

Molecular quantification of bacteria from respiratory samples in patients with suspected ventilator-associated pneumonia

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ABSTRACT

Ventilator-associated pneumonia (VAP) is the most common infection in critically ill patients. Initial antibiotic therapy is often broad spectrum, which promotes antibiotic resistance so new techniques are under investigation to obtain early microbiological identification and quantification. This trial compares the performance of a new real-time quantitative molecular-based method with conventional culture in patients with suspected VAP. Patients with suspected VAP who were ventilated for at least 48 h were eligible. An endotracheal aspirate (ETA) and a bronchoalveolar lavage (BAL) were performed at each suspected VAP episode. Both samples were analysed by conventional culture and molecular analysis. For the latter, bacterial DNA was extracted from each sample and real-time PCR were run. In all, 120 patients were finally included; 76% (91) were men; median age was 65 years, and clinical pulmonary infection score was ≥6 for 73.5% (86) of patients. A total of 120 BAL and 103 ETA could be processed and culture results above the agreed threshold were obtained for 75.0% (90/120) of BAL and 60.2% (62/103) of ETA. The main isolated bacteria were *Staphylococcus aureus*, *Pseudomonas aeruginosa* and *Haemophilus influenzae*. Performance was 89.2% (83.2%–93.6%) sensitivity and 97.1% (96.1%–97.9%) specificity for BAL samples and 71.8% (61.0%–81.0%) sensitivity and 96.6% (95.4%–97.5%) specificity for ETA samples when the molecular biology method was compared with conventional culture method (chosen as reference standard). This new molecular method can provide reliable quantitative microbiological data and is highly specific with good sensitivity for common pathogens involved in VAP. **M. Clavel, CMI 2016;22:812.e1–812.e7**

Introduction

Ventilator-associated pneumonia (VAP) is the most common nosocomial infection in critically ill patients. Between 10% and 20% of the patients receiving more than 48 h of mechanical ventilation will develop VAP [1]. The actual mortality rate of VAP is still controversial but can exceed 50% when the initial therapy is not appropriate [2]. Antibiotic therapy is based on the presence of risk factors for infection with multidrug-resistant pathogens and is often broad spectrum [3]. Nevertheless, this approach mandates de-escalation of therapy once microbiological data become available, to prevent antimicrobial resistance and drug toxicity [4,5]. Obtaining the identification but also the quantification of bacteria responsible for VAP within a couple of hours would allow an earlier effective and targeted antibiotic treatment. The purpose of this prospective multicentre feasibility trial was to compare the performance of a new real-time quantitative molecular-based protocol with conventional microbiological culture in patients with suspected VAP.

Materials and methods

Study design

The study was a proof-of-concept, prospective, observational, non-randomized, multicentre clinical trial conducted between

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January and November 2013 at the following four French sites: University Hospital of Limoges, General Hospital of Brive, University Hospital of Tours and General Hospital of Angoulême. Intensive care unit patients who were intubated and had received mechanical ventilation for at least 48 h were eligible for the study if they met all the following criteria: (a) older than 18 years, (b) clinical suspicion of VAP defined by a new or progressive infiltrate on chest radiography associated with at least two of the following criteria: core temperature >38.3°C or <35.0°C, leucocyte count ≥10 000/μL or ≤4500/μL and new onset of purulent tracheal secretions. Patients were excluded if they (a) had severe hypoxaemia (Pao_2/Fio_2 <100), (b) were immunocompromised or neutropenic, or (c) had refractory septic shock.

Ethics statement

The study was conducted in accordance with the declaration of Helsinski and International Council for Harmonization Good Clinical Practice. The ethics committee of Limoges hospital accepted the study and informed consent was not required. However, written information was given to the patients or their legally authorized representative before enrolment as required by French law.

Samples collection

On the day of suspected VAP, a bronchoalveolar lavage (BAL) sample was collected preceded by an endotracheal aspirate (ETA) sample collected by nurses (a catheter was inserted 30 cm into the endotracheal tube and the specimen was suctioned under aseptic conditions). BAL collection was standardized between the four centres according to a previously described technique [6]. A video bronchoscope was introduced via an intratracheal tube (7.5 or 8 mm) in the breathing duct through an extension tube (DAR-Covidien; Covidien, Boulder, CO, USA). BAL were performed by successive introduction of five aliquots of 20 mL of salted isotonic serum (37°C) in the segment or lobe in which the new or progressive infiltration had developed. Each aliquot was carefully suctioned before the administration of the next one, the first being discarded for microbiological analysis. A part of both samples, BAL and ETA, was used for conventional microbiological analysis and the remainders were aliquoted and frozen at −80°C for molecular analysis.

Laboratory methods

Molecular biology-based method

DNA was extracted in batches (11 specimens and the 11 reference strains required for quantification at the same time). Each extraction batch lasted around 2 h 30 min for BAL and around 3 h 15 min for ETA.

Before any processing, a precise quantity of *Staphylococcus pseudintermedius* (1500 CFU for BAL, 7500 CFU for ETA), used as Specimen Processing Control (SPC), was added to all specimens. Each BAL specimen (500 μL) was incubated with 5 μg lysin (Hyglos GmbH, Bernried am Starnberger See, Germany) for 15 min at 37°C before nucleic acid extraction. For ETA specimens, liquefaction and pre-processing were performed as previously described [7]. DNA was extracted from all lysates using the NucliSENS® easyMag® (bioMérieux SA, Marcy l'Etoile, France), according to the specific B2.0.1 protocol, with an elution volume of 55 μL.

Real-time PCR were run in batches for each pathogen independently. Primers and probes were used to detect the SPC and the main pathogens involved in VAP (see Supplementary material, Table S1): *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Haemophilus influenzae*, *Proteus mirabilis*, *Escherichia coli*, *Serratia marcescens*, *Streptococcus pneumoniae*, *Acinetobacter baumannii*, *Enterobacter aerogenes*, *Klebsiella pneumoniae* and *Klebsiella oxytoca*. A 'pan-Enterobacteria' real-time quantitative PCR (qPCR) that targeted almost all species belonging to the *Enterobacteriaceae* family was also used. The qPCR were run on the Applied Biosystems® 7500 Fast Dx Real-Time PCR Instrument (Open version; Life Technologies, Waltham, MA, USA). Each qPCR was performed by using 2 μL of the NucliSens easyMAG® eluate, using the FastStart *Taq* DNA polymerase kit as recommended by the supplier (Roche, Basel, Switzerland). A cycle threshold (Ct) of 34 (± 1 Ct) was expected for the SPC target.

Quantifications were performed using qPCR standard curves, by converting Ct to CFU/mL. Briefly, for each pathogen, 100 μL of a calibrated McFarland solution of a reference strain (representing a bacterial quantity of approximately 10^7 CFU and determined precisely by plating dilutions) was transferred into a tube containing 400 μL 0.9% NaCl for BAL standards, or 300 μL of a solution previously described [7]. These reference strains were either obtained from an external provider (ATCC, LGC Standards, Molsheim, France) or from bioMérieux's internal biobank. Identification was confirmed for all of them using the Vitek® MS system (bioMérieux). Artificial specimens were submitted to the whole process in parallel to the clinical specimens, and each easyMAG® eluate was serially diluted to build a standard curve in the qPCR plate. *Escherichia coli* was used as reference strain to build the Enterobacteria qPCR standard curve. As done for the conventional culture, a significant sample was defined as at least one pathogen load $\geq 10^4$ CFU/mL for BAL and $\geq 10^6$ CFU/mL for ETA.

The method was validated internally (limits of detection, limits of quantification and robustness), for each pathogen individually (data not shown). Limits of detection were ~10^2 CFU/mL, lower limits of quantification were ~10^3 CFU/mL and upper limits of quantification were ~10^7 CFU/mL.

Conventional methods

Microbiological analyses of BAL and ETA specimens were performed according to current recommendation of the European Manual of Clinical Microbiology [8]. ETA were treated with Digest-EUR (V/V) (Eurobio, Courtaboeuf, France) and mixed by vortex with glass beads (bioMérieux). One hundred microlitres was diluted into 1 mL of sterile saline solution and 10 μL of this suspension was seeded on non-selective (cystine lactose electrolyte deficient; COS) and selective (Chocolate agar; Haem) agar plates (bioMérieux SA, Mrcy l'Etoile, France) incubated at 37°C for 48 h. Dilutions were performed so as to obtain a detection threshold of 10^3 CFU/mL. No liquefaction step was performed for BAL: 10 μL of raw BAL were directly seeded on the same agar plates with a detection threshold of 10^2 CFU/mL. Bacterial identifications were performed using the Vitek2® system, the Vitek® MS system and/or API® kits (bioMérieux). A significant culture was defined as at least one pathogen load $\geq 10^4$ CFU/mL for BAL and $\geq 10^6$ CFU/mL for ETA.

Statistical methods

The study was designed to assess the concordance between the quantification of the bacterial loads present in BAL samples obtained by the qPCR and by conventional culture. It was based on the targeted performance, the prevalence of the three main expected pathogens (*S. aureus*, *P. aeruginosa* and *H. influenzae*) in the study sites and a global associated risk α at 5%. A minimum of 120 patients in the intensive care units with suspected VAP had to be included. Bacterial loads of the three main expected pathogens present in the BAL samples (and ETA when available) were quantified using the standard method and independently using the molecular-based biology prototype. For information only, concordance was also estimated for the other species detected by the PCR assay.