

Peptide nucleic acid fluorescence *in situ* hybridization for rapid detection of *Klebsiella pneumoniae* from positive blood cultures

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This study evaluated a novel peptide nucleic acid (PNA) probe targeting a region of the 23S rRNA gene of *Klebsiella pneumoniae* by fluorescence *in situ* hybridization (FISH). Analytical performance was determined using 39 reference strains and other well-characterized strains of *Klebsiella* spp. and *Enterobacter aerogenes*. The probe was found to be specific for the *K. pneumoniae* complex (*K. pneumoniae* including *Klebsiella ozaenae* and *Klebsiella variicola*). The diagnostic accuracy was evaluated with 264 blood cultures containing Gram-negative rods. Using conventional identification as the reference, performance specifications were as follows: sensitivity 98.8 %, specificity 99.5 %, positive predictive value 98.8 % and negative predictive value 99.5 %. Discrepancies were resolved by PNA FISH retest and phenotypic tests. In conclusion, the *K. pneumoniae* probe provided an accurate diagnosis within 3 h and may supplement other methods for direct identification of Gram-negative bacteria.

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INTRODUCTION

Bacteraemia has an overall in-hospital mortality of above 20 % (Laupland *et al.*, 2004; Panceri *et al.*, 2004; Pedersen *et al.*, 2003), and rapid, efficient and accurate detection of bacteraemia is a critical task of clinical microbiology laboratories. Conventional culture-based techniques are time-consuming and there is a demand for methods that are directly applicable to positive blood cultures (BCs).

After *Escherichia coli*, *Klebsiella pneumoniae* is the most common cause of Gram-negative bacteraemia and within its genus it accounts for three-quarters of all isolates, with some local variations (Feldman *et al.*, 1990; Hansen *et al.*, 1998). There is some controversy around the best way of classifying members of the genus *Klebsiella*, and as definitive identification by conventional methods may be cumbersome (Hansen *et al.*, 2004), new methods for accelerated diagnosis would be advantageous.

Fluorescence *in situ* hybridization (FISH) with peptide nucleic acid (PNA) probes is a novel technique using fluorescein-labelled PNA probes targeting rRNA genes for rapid diagnosis of major BC pathogens (Oliveira *et al.*, 2002; Rigby *et al.*, 2002; Sogaard *et al.*, 2005). The objectives of this study were to (i) evaluate a new PNA probe targeting *K. pneumoniae* using a panel of well-characterized strains representing *Klebsiella* spp. and the closely related *Enterobacter aerogenes*, and (ii) determine the utility of this assay for rapid identification of *K. pneumoniae* in routinely processed BCs.

METHODS

Study setting. This observational study was conducted from May 2003 to April 2004 and from January 2005 to July 2005 at the Department of Clinical Microbiology, Aalborg Hospital, Denmark. The department provides diagnostic bacteriology including BCs for one referral hospital and six district hospitals (approx. 1800 hospital beds) in the County of North Jutland, Denmark. In the initial period, we collected the slides prospectively as a part of a separate study

Abbreviations: BC, blood cultures; FISH, fluorescence *in situ* hybridization; PNA, peptide nucleic acid.

(Søgaard *et al.*, 2005) during which the *K. pneumoniae* probe was not available. In order to increase the proportion of non-*Escherichia coli* Gram-negative rods in the sample, we included only one-tenth of the BCs that were positive for *Escherichia coli*.

Type strains and well-characterized clinical isolates. Type strains for the following species and subspecies were included in the evaluation: ATCC 13883 (*K. pneumoniae*), NCTC 5050 (*Klebsiella ozaenae*), ATCC 13182 (*Klebsiella oxytoca*), ATCC 33257 (*Klebsiella terrigena*), ATCC 33531 (*Klebsiella planticola*), JCM 6096 (*Klebsiella ornithinolytica*) and NCTC 10006 (*Enterobacter cloacae*). We also obtained the following panel of well-characterized clinical isolates from the collection at the International Escherichia and Klebsiella Reference Center (WHO) (Statens Serum Institut, Copenhagen, Denmark): *K. pneumoniae* ($n=5$), *K. ozaenae* ($n=1$), *K. oxytoca* ($n=5$), *Klebsiella variicola* ($n=1$), *K. terrigena* ($n=5$), *K. planticola* ($n=5$), *K. ornithinolytica* ($n=5$) and *Enterobacter cloacae* ($n=5$). Strains were biochemically characterized (Hansen *et al.*, 2004) and additional sequencing of the 16S rRNA gene or the *gyrA* gene was performed (Boye & Hansen, 2003; Brisse & Verhoef, 2001).

All strains were grown by inoculating 0.1 ml of a 10^3 – 10^4 c.f.u. ml⁻¹ bacterial suspension in routine aerobic FAN BC bottles with a negative growth index after 7 days' incubation. Bottles were loaded promptly into the BacT/Alert BC system and incubated until flagged positive and then unloaded and handled as described for the *K. pneumoniae* PNA FISH method. Reculture and retesting was performed for all positive reactions and six randomly chosen strains with negative reactions; otherwise strains were tested only once.

BC isolates. A BC set comprised one standard aerobic bottle, one FAN aerobic bottle and one anaerobic standard bottle for adult patients. An aerobic paediatric FAN bottle was used for children under the age of 6 years. Positive BCs were examined by direct microscopy and Gram staining, and cultures with Gram-negative rods were included as long as only one morphological type was observed. Only one bottle per BC set was included; if two or three bottles in a set were positive, the FAN bottle was selected ($n=166$); otherwise the anaerobic bottle ($n=50$) or the standard aerobic bottle ($n=38$) was selected, in that order. Ten negative BCs were included in the analysis as negative controls.

A total of 264 BCs with Gram-negative rods were included: *K. pneumoniae* (including one *K. pneumoniae* subsp. *ozaenae*), 80; *K. oxytoca*, 35; *K. planticola*, 1; *Escherichia coli*, 34; *Citrobacter* spp., 8; *Enterobacter* spp., 14; *Morganella morganii*, 5; *Proteus* spp., 11; *Serratia* spp., 6; *Yersinia* sp., 1; *Salmonella* spp., 9; *Moraxella* sp., 1; *Capnocytophaga* sp., 1; *Haemophilus* spp., 11; *Acinetobacter* spp., 7; *Agrobacterium* spp., 2; *Burkholderia* sp., 1; *Pseudomonas* spp., 16; *Stenotrophomonas maltophilia*, 2; unidentified aerobic Gram-negative rod, 1; *Bacteroides* spp., 15; *Fusobacterium* sp., 2; unidentified anaerobic Gram-negative rod, 1.

PNA FISH. The PNA probe was synthesized by Applied Biosystems. The probe sequence (5'-Flu-OO-CACCTACACACCAGC-3', where Flu=5,6-carboxyfluorescein and O=8-amino-3,6-dioxaoctanoic acid) is directed against a region of the 23S rRNA gene of *K. pneumoniae* starting at nt 1704 (Ludwig *et al.*, 1995). The probe was designed to take advantage of a region of sequence heterogeneity between *K. pneumoniae* and *K. oxytoca* 23S rRNA genes, similar to a previously published DNA probe (Kempf *et al.*, 2000).

The PNA FISH assay was performed according to the manufacturer's instructions (Oliveira *et al.*, 2002). In brief, 10 µl BC broth was mixed on one-well Teflon-coated microscope slides (AdvanDx) with a drop of fixation solution, air-dried and fixed by flame fixation. Slides not examined immediately were stored at room temperature in the dark. One drop of hybridization solution containing the fluorescein-labelled

PNA probe was added and a coverslip was applied. For hybridization, slides were incubated on a slide warmer (SM30; Grant Boekel) at 55 °C for 90 min. Subsequently, coverslips were removed and the slides were submerged for 30 min in a pre-heated wash solution (55 °C) in a water bath. Slides were mounted with mounting fluid (KT001; AdvanDx) and coverslips were applied before examination under a fluorescence microscope ($\times 100$ objective, Olympus BX40; Osram HBO 100 W/2 Hg lamp) equipped with a fluorescein isothiocyanate–Texas red dual band-pass filter (filter no. AC003; AdvanDx).

Two observers who were blind to the results by conventional identification examined the slides. Distinct green fluorescence of multiple bacteria was scored as a positive result and graded as bright or faint. Negative slides had a faintly reddish background; rarely, a few fluorescent bacteria or bacterial clusters were observed, but lower fluorescence intensity and cell numbers clearly distinguished these from the positive results.

Conventional identification. We performed subculture on 5% horse blood agar, chocolate agar, CPS2 agar (bioMérieux) and K vitamin-enriched chocolate agar for anaerobic culture, as deemed appropriate. Blood culture isolates were identified tentatively on the basis of colony morphology and Gram staining. The CPS2 agar facilitated diagnostic shortcuts for typical *Escherichia coli* (β -glucuronidase-positive, indicated by burgundy-coloured colonies, and spot indole-positive) and swarming *Proteus* spp. (tryptophan deaminase-positive and spot indole-positive). All other enterobacteria were identified with the ID-GNB card in the Vitek2 system (bioMérieux); a negative spot indole test was required for the identification of *K. pneumoniae*.

The results obtained by conventional identification were used as reference. If a discrepancy between conventional identification and PNA FISH was observed, the isolate was retested by use of simulated culture and referred to the International Escherichia and Klebsiella Reference Center (WHO) (Statens Serum Institut) for extensive biochemical testing (Hansen *et al.*, 2004).

The performance characteristics of the *K. pneumoniae* probe were evaluated by calculation of the sensitivity, specificity and positive and negative predictive values.

RESULTS AND DISCUSSION

Analytical performance

The probe was specific for *K. pneumoniae* including the biotype *K. ozaenae* and the closely related *K. variicola*. Faint positive reactions obtained for 3/6 *K. ornithinolytica* isolates and 1/6 *K. planticola* isolates could not be reproduced.

We included *Enterobacter aerogenes* (a valid synonym of *K. mobilis*), as this species, based on phenotypic traits and DNA relatedness, is more closely related to *Klebsiella* spp. than to *Enterobacter cloacae* (the type species of the genus *Enterobacter*) (Bascomb *et al.*, 1971; see also discussion by Boye & Hansen, 2003). *K. variicola* ('adonitol non-fermenting *K. pneumoniae*') appears to correspond to the KpIII cluster of *K. pneumoniae* (Brisse *et al.*, 2004) and we therefore regard both *K. ozaenae* and *K. variicola* as members of the *K. pneumoniae* complex.

Among the species included, only *K. pneumoniae*, *K. oxytoca* and *Enterobacter aerogenes* are frequently reported

BC isolates. The lack of easily determinable and distinct phenotypic characteristics within the genus *Klebsiella* is a limitation of commercial identification systems and, as a consequence, the clinical importance of other *Klebsiella* spp. is known only sporadically (Liu *et al.*, 1997; Podschun *et al.*, 2001; Rosenblueth *et al.*, 2004; Westbrook *et al.*, 2000). Seen in this perspective, easy identification of *K. pneumoniae*, *K. ozaenae* and *K. variicola* by PNA FISH could be helpful.

Diagnostic performance

Seventy-nine out of 82 Gram-negative rods conventionally identified as *K. pneumoniae* were positive by PNA FISH and three were negative. When re-examined by extensive biochemical tests, two of the three isolates were found to be *K. oxytoca* and *K. planticola*. The last false negative yielded a bright positive result upon retesting with PNA FISH.

Among 184 Gram-negative rods not identified as *K. pneumoniae*, 182 were negative by PNA FISH and two were positive. One discrepant culture, which stained only faintly and was negative by PNA FISH when retested, was identified as *Pseudomonas aeruginosa*. The other discrepant culture was initially identified as *Klebsiella* sp., but further biochemical testing showed it to be *K. pneumoniae*. Thus we obtained only one false-positive result.

Based on these data, the performance specifications for the *K. pneumoniae* PNA FISH assay were: diagnostic sensitivity 98.8% (79/80 specimens), diagnostic specificity 99.5% (183/184 specimens), positive predictive value 98.8% (79/80 specimens) and negative predictive value 99.5% (183/184 specimens). These estimates are likely to be conservative because of the low proportion of *Escherichia coli* isolates included in our study and because all *Escherichia coli* isolates were uniformly negative.

Except for this under-representation of *Escherichia coli*, the study was conducted in a routine setting and the few spurious results that occurred might have been due to handling problems in the laboratory. The preparatory steps prior to hybridization could be critical, as a false-negative *K. pneumoniae* isolate was positive upon retest and the false-positive *P. aeruginosa* isolate was negative when retested.

Our results corroborate previous studies with PNA FISH assays targeting other microbial pathogens (Oliveira *et al.*, 2002; Rigby *et al.*, 2002; Søgaard *et al.*, 2005). PNA probes are known to have an overall superior performance to DNA probes when used in the FISH format, including greater specificity (Wilks & Keevil, 2006). The absence of complete sequence information for the rarer *Klebsiella* spp. argued for selection of the more-specific (PNA) probes in this study.

K. pneumoniae, *Escherichia coli* and *P. aeruginosa* are the major Gram-negative BC pathogens and the combined use of probes targeting these would allow rapid and specific identification of nearly two-thirds of Gram-negative bacteraemias. In countries like Denmark, where most

bacterial groups still have a predictable pattern of resistance, such a diagnostic strategy could support early directed antibiotic therapy.

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