

Role of *Burkholderia pseudomallei* biofilm formation and lipopolysaccharide in relapse of melioidosis

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Abstract

We examined whether quantitative biofilm formation and/or lipopolysaccharide type of *Burkholderia pseudomallei* was associated with relapsing melioidosis. We devised a 1 : 4 nested case-control study in which both cases and controls were drawn from a cohort of patients with primary melioidosis. Paired isolates from 80 patients with relapse and single isolates from 184 patients without relapse were tested. Relapse was associated with biofilm formation of the primary infecting isolate (conditional OR 2.03; 95% CI 1.27–3.25; p 0.003), but not with lipopolysaccharide type (p 0.74). This finding highlights the importance of biofilm formation in relapsing melioidosis.

Keywords: Biofilm, lipopolysaccharide, melioidosis, *pseudomallei*, relapse

Original Submission: 25 January 2014; **Revised Submission:** 26 February 2014; **Accepted:** 1 March 2014

Editor: S. Cutler

Article published online: 7 March 2014

Clin Microbiol Infect 2014; **20**: O854–O856

10.1111/1469-0691.12614

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Introduction

Melioidosis, an infectious disease caused by the Tier I select agent *Burkholderia pseudomallei*, is notoriously difficult to cure [1]. North-eastern Thailand is a hot spot for this infection, with an annual incidence of 21.0 per 100 000 population and a case-fatality rate of 40% [2]. For patients who survive their first episode of infection, the single most important complication is recurrent melioidosis following apparent cure. This occurs in approximately 13% of patients followed for 10 years, and half of the recurrences occur within 12 months of the primary episode [3]. Approximately one-quarter of those with relapse will die as a direct result [4,5].

Biofilm formation has been described as an important factor associated with persistent infections in a number of infectious diseases, including *Burkholderia cepacia* infection in cystic fibrosis patients [6–8], but this has not been formally evaluated in relation to relapse of melioidosis. A previous study of a small number of isolates associated with relapse suggested that *B. pseudomallei* with uncommon lipopolysaccharide (LPS) types (smooth type B and rough type) might be associated with relapse [9]. Here, we evaluated biofilm formation and LPS type of *B. pseudomallei* isolates from patients with primary melioidosis drawn from a cohort described previously [5], and determined their associations with relapse.

We devised a 1 : 4 nested case-control study in which both cases and controls were drawn from a cohort of patients with primary melioidosis identified between 1986 and 2004 who survived to receive oral antimicrobial therapy and were observed until July 2005 [5]. Cases were all patients who developed at least one episode of relapse during the study period, with relapse being verified by genotyping of the primary and relapse isolates [5]. Controls were randomly selected from those patients in the cohort who had not developed relapse by the time relapse was identified in cases. Cases and controls were matched for known risk factors for relapse, including choice and duration of oral antimicrobial therapy, positive blood culture, and multifocal distribution of infection at first presentation. Primary isolates from cases and controls and relapse isolates from cases were evaluated for quantitative biofilm formation and LPS. All isolates were stored at -80°C prior to the evaluation. The first isolate cultured and saved from each episode was used in the study. Quantitative estimation of biofilm formation was performed with a modified microtitre plate test, as described previously [10–12]. All experiments were independently conducted twice, and the results reported were the average from those two exper-

iments. LPS was extracted, and the type was defined as smooth type A, smooth type B, or rough type, as described previously [9]. A conditional logistic regression model was used to evaluate the relationships between independent factors and the relapse outcome. Selection of controls and statistical analyses were conducted with STATA, version 12.0 (Stata-Corp, College Station, TX, USA). The study was approved by the Ethical and Scientific review subcommittee of the Thai Ministry of Public Health [5].

Of 86 and 202 primary isolates from cases and matched controls, 80 (93%) and 184 (91%), respectively, were available for study and included in the analysis. Patients in the case and control groups had comparable characteristics for the matched variables (Table 1).

First, we determined whether the quantitative production of biofilm by the primary isolate influenced the likelihood that relapse would occur. Biofilm production by primary isolates from patients with relapse was higher than that for primary isolates from matched patients without relapse (median corrected optical density (OD)_{630 nm} of 0.95 (interquartile range 0.75–1.28) vs. 0.79 (interquartile range 0.63–1.06)). This was independently associated with the relapse outcome (conditional OR 2.03; 95% CI 1.27–3.25; *p* 0.003). Overall, 99% of primary isolates from cases and 98% from controls had

LPS smooth type A. LPS smooth type B was found in one (1%) case and in two (1%) controls, and rough-type LPS was found in one (1%) control. An association between LPS type of the primary isolate and relapse was not found (*p* 0.74).

Next, we determined whether there was any difference in biofilm formation and in LPS type between primary and relapse isolates from the same relapse cases. From 80 relapse cases, 71 (89%) paired primary–relapse isolates were available and evaluated. Biofilm formation of the primary isolate and that of the relapse isolate were not different (mean difference for corrected OD_{630 nm} of 0.002; 95% CI –0.16 to 0.16; *p* 0.98). The LPS type of the primary–relapse pair was the same for all 71 isolates (LPS smooth type A, *n* = 70; LPS smooth type B, *n* = 1).

In this study, we have shown that a quantitative measure of biofilm formation by the primary isolate is associated with relapse in patients with melioidosis. This was independent of known clinical risk factors for relapse, including choice and duration of oral antimicrobial therapy, positive blood culture, and multifocal distribution of infection at first presentation, factors that were matched by the nested case–control study design. This provides the first evidence to suggest that biofilm formation of *B. pseudomallei in vitro* is associated with relapse in human melioidosis, and is consistent with findings reported for *Escherichia coli* [6] and other biofilm-producing bacteria [7,8]. We also observed that quantitative biofilm formation did not differ between paired primary and relapse isolates. This lack of detectable change between isolates of the same lineage that are separated by the period spanning human infection, quiescence and re-emergence argues against the notion that increased biofilm formation occurs *in vivo* through positive selection.

Quantitative biofilm formation by isolates in this study was lower overall than that reported previously for 34 clinical *B. pseudomallei* isolates (mean corrected OD_{630 nm} of 1.98 ± 0.32) [12]. Possible explanations are that the means used in the previous study were skewed by isolates with exceptionally high biofilm formation. In addition, the isolates in the study described here were only from patients who survived the first episode of acute infection, whereas the isolates in the previous study included those who died during the acute infection.

The majority of *B. pseudomallei* isolates from the primary episode of melioidosis in this study expressed LPS smooth type A, and no association between LPS type and relapse was found. This contrasts with the findings of a previous study, in which three of 11 (27%) patients with recurrent melioidosis had different LPS types in the primary and relapse isolates [9]. The finding in the previous study may relate to a small sample size. Furthermore, isolates in this previous study were not genotyped, and the possibility that recurrence was attributable to re-infection with a different isolate rather than persistence

TABLE 1. Characteristics of cases and controls

Characteristics	Cases N = 80	Controls N = 184	p-Value ^a
Median age in years (interquartile range)	49 (41–58)	47 (38–57)	0.47
Male sex, <i>n</i> (%)	53 (66)	104 (56)	0.32
Underlying diseases, <i>n</i> (%)			
Diabetes mellitus	49 (61)	103 (56)	0.24
Renal calculi	14 (18)	21 (11)	0.43
Distribution of melioidosis ^b , <i>n</i> (%)			
Localized	27 (34)	64 (35)	NA
Multifocal	13 (16)	29 (16)	
Bacteraemic	23 (29)	56 (30)	
Disseminated	17 (21)	35 (19)	
Site or organ(s) infected during the primary episode, <i>n</i> (%)			
Bacteraemia	40 (50)	91 (49)	NA
Pneumonia	30 (38)	75 (41)	0.84
Liver abscess	24 (30)	45 (24)	0.28
Splenic abscess	29 (36)	54 (29)	0.50
Septic arthritis	8 (10)	19 (10)	0.67
Osteomyelitis	2 (3)	4 (2)	0.85
Biofilm formation, corrected OD _{630 nm} (interquartile range)	0.95 (0.75–1.28)	0.79 (0.63–1.06)	0.003
Biofilm, <i>n</i> (%)			
Smooth type A	79 (99)	181 (98)	0.74
Smooth type B	1 (1)	2 (1)	
Rough type	–	1 (1)	

NA, not applicable, as choice and duration of oral antimicrobial therapy, positive blood culture result and multifocal distribution of first presentation were matched variables; OD, optical density.

^ap-Values were calculated with a conditional logistic regression model.

^bMelioidosis was classified as localized (single focus of infection and a negative blood culture result), multifocal (one or more non-contiguous foci of infection and a negative blood culture result), bacteraemic (a positive blood culture result plus a single or no identifiable focus of infection), and disseminated (a positive blood culture result plus one or more non-contiguous foci of infection).

and relapse with the primary isolate was not excluded [9]. The proportions of uncommon, non-type A LPS in the primary episode of melioidosis in this study (1% in cases and 2% in controls) were similar to those observed in the previous study (3%) [9]. Our findings, based on a much larger number of bacterial isolates supported by a robust study design, do not provide evidence for a link between uncommon, non-type A LPS and relapse.

Acknowledgements

We are grateful to the staff of the Wellcome Trust–Oxford University–Mahidol University Tropical Medicine Research Programme and the Melioidosis Research Centre, Khon Kaen University, Thailand.

Funding

This study was supported by the Thailand Research Fund (MRG5480172) and the Melioidosis Research Centre, Khon Kaen University, Thailand. D. Limmathurotsakul was supported by a Wellcome Trust project grant (090219/Z/09/Z). S. J. Peacock was supported by the National Institute for Health Research Cambridge Biomedical Research Centre.

Transparency Declaration

All authors have no conflict of interest.

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