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Recurrent Melioidosis in Patients in Northeast Thailand Is Frequently Due to Reinfection Rather than Relapse

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Human melioidosis is associated with a high rate of recurrent disease, despite adequate antimicrobial treatment. Here, we define the rate of relapse versus the rate of reinfection in 116 patients with 123 episodes of recurrent melioidosis who were treated at Sappasithiprasong Hospital in Northeast Thailand between 1986 and 2005. Pulsed-field gel electrophoresis was performed on all isolates; isolates from primary and recurrent disease for a given patient different by one or more bands were examined by a sequence-based approach based on multilocus sequence typing. Overall, 92 episodes (75%) of recurrent disease were caused by the same strain (relapse) and 31 episodes (25%) were due to infection with a new strain (reinfection). The interval to recurrence differed between patients with relapse and reinfection; those with relapses had a median time to relapse of 228 days (range, 15 to 3,757 days; interquartile range [IQR], 99.5 to 608 days), while those with reinfection had a median time to reinfection of 823 days (range, 17 to 2,931 days; IQR, 453 to 1,211 days) ($P = 0.0001$). A total of 64 episodes (52%) occurred within 12 months of the primary infection. Relapse was responsible for 57 of 64 (89%) episodes of recurrent infection within the first year after primary disease, whereas relapse was responsible for 35 of 59 (59%) episodes after 1 year ($P < 0.0001$). Our data indicate that in this setting of endemicity, reinfection is responsible for one-quarter of recurrent cases. This finding has important implications for the clinical management of melioidosis patients and for antibiotic treatment studies that use recurrent disease as a marker for treatment failure.

Melioidosis is a severe infection caused by the gram-negative bacillus *Burkholderia pseudomallei*, an environmental saprophyte present in Southeast Asia and northern Australia (17). A major feature of human disease is that bacterial eradication is difficult to achieve. The clinical response to intravenous antibiotics is slow, with a median fever clearance time in our patient population of 8.5 days. Although prolonged courses of antibiotic treatment (at least 10 days of intravenous antibiotics, followed by 12 to 20 weeks of oral antibiotics) are recommended, recurrent disease is common (at a rate of ≥6% in the first year) (2, 5). A prolonged period of dormancy of up to 62 years may also occur between a presumed exposure to *B. pseudomallei* and clinical manifestations of infection (9).

Bacterial mechanisms and host susceptibility for recurrent melioidosis are poorly understood. However, the ability of *B. pseudomallei* to survive in the human host supports the idea that recurrence is more likely to be associated with a relapse caused by bacteria persisting within a sequestered focus than to reinfection with a different strain. This is consistent with previous small studies in which isolates from the first and second episodes of infection in a given patient usually had the same genotype (5, 6, 14). Here, we report the results of a large

genotyping study of isolates from 116 patients in Northeast Thailand with 123 episodes of recurrent melioidosis.

MATERIALS AND METHODS

Patients and bacterial isolates. Patients were prospectively recruited by a study team based at Sappasithiprasong Hospital, Ubon Ratchathani, northeast Thailand, between September 1986 and January 2005. Patients with suspected melioidosis were actively sought during twice-daily rounds of the medical and intensive care wards, together with passive surveillance of surgical and pediatric wards. Detailed clinical information was recorded, including a documented history of culture-proven melioidosis. Blood and throat swab samples were collected from all patients for culture; other specimens (urine, pus, and surface swabs of skin lesions) were collected where available and were processed by standard methods (16). Isolates were stored in Trypticase soy broth with 15% glycerol at −80°C.

PFGE. For all patients with more than one episode of disease, the first *B. pseudomallei* isolates cultured on the first and subsequent episodes of melioidosis were examined by pulsed-field gel electrophoresis (PFGE). A single bacterial colony was streaked onto Columbia agar and incubated for 48 h at 37°C in air. The colonies were harvested and suspended to an optical density at 540 nm of 0.7 in suspension buffer (75 mM sodium chloride, 25 mM EDTA, pH 7.5). This was mixed (1:1) with molten 2% low-melting-point agarose (Ultrapure; Gibco) and pipetted into PFGE plug molds (Bio-Rad Laboratories, Hercules, CA). The plugs were lysed overnight at 56°C in lysis buffer (0.1% sodium dodecyl sulfate, 25 mM EDTA, pH 8.0) containing 500 μg/ml proteinase K (Invitrogen) and then rinsed three times with TE buffer (10 mM Tris, 10 mM EDTA). Prior to PFGE, the plugs were digested overnight with 10 U SpeI (New England Biolabs) at 37°C before being loaded into a 1% agarose gel (Gibco) in 0.5× TBE buffer (Tris-borate-EDTA). Each well was overlaid with 0.8% low-melting-point agarose. PFGE was performed on a CHEF-DRIII system (Bio-Rad Laboratories, Richmond, CA) for 44 h at a temperature of 14°C and at 6 V/cm using the following parameters: initial switch time, final switch time, and run time for block I, 10 to

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60 V for 18 h; those for block II, 30 to 40 V for 18 h; and those for block III, 50 to 90 V for 8 h. Bacteriophage lambda concatemers were run as the standard (Promega). Gels were stained with ethidium bromide, washed in water, and photographed under UV light by using the Gel Doc 1000 system (Bio-Rad). Analysis of the PFGE banding patterns was performed with BioNumerics (version 2.5) software (Applied Maths, Belgium).

MLST. Multilocus sequence typing (MLST) was performed as described previously (7), with the exception that *gmhD* was amplified by using a nested approach with the following primers: outer primer pair, gmhD-up (5′-TCGCG CAGGGCACGCAGTT) and gmhD-dn (GGCTGCCGACCGTGAGACC); inner primer pair, gmhD-up (5′-TCGCGCAGGGCACGCAGTT) and gmhD-dn (GTCAGGAACGGCGCGTCGTAGC). Alleles at each of the seven loci were assigned, and the allelic profile (presented as a string of seven integers) was used to define the sequence type (ST) using the *B. pseudomallei* MLST website (http://bpseudomallei.mlst.net/).

Colony morphology. The appearance of the colony morphology was examined for use as a guide to the likelihood of reinfection. Isolates were spread plated to achieve single colonies on Ashdown's agar and incubated for 4 days at 37°C in air. The colony morphologies were compared between the first and subsequent cultures for each patient.

Definitions and analysis. Recurrent disease was defined as clinical features of melioidosis in association with one or more cultures positive for *B. pseudomallei* in a patient with a history of one or more previous episodes. This included patients who had completed treatment for the previous episode, together with patients who had recurrent symptoms while receiving oral antibiotics for melioidosis following clear initial clinical and microbiological responses to antibiotic therapy.

Disease relapse and reinfection were defined on the basis of the typing of the isolates from the first episode and the subsequent episode(s). Isolates from the same patient with identical banding patterns on PFGE were considered to represent a single isolate, and these patients were classified as having relapse. Isolates from the same patient that differed by one or more bands were examined using a screening approach based on MLST. This used the assumption that a difference in just one of seven MLST loci is sufficient to assign different sequence types. Two of the seven MLST loci, *narK* and *gmdD*, are variable within the bacterial population. The sequence of *narK* was first determined, followed by sequencing of *gmhD* for isolate pairs with the same allele *narK*. Isolates from the same patient that differed at one or both of these loci were considered to represent reinfection. Those with sequence identity at both *narK* and *gmhD* were further characterized using the remaining five MLST loci to define the full sequence type.

Statistical tests were performed using Intercooled Stata 8.0 software (Stata Corporation, College Station, TX). Proportions were compared by Fisher's exact test, and continuous variables were compared by the Wilcoxon rank-sum test. Ethical approval for clinical studies at Ubon Ratchathani has been obtained from the Ministry of Public Health, Royal Government of Thailand.

RESULTS

A total of 130 patients presented with 145 episodes of a culture-proven recurrence of melioidosis between September 1986 and January 2005. Of these, four patients had three episodes of recurrence, seven patients had two episodes of recurrence, and the remainder had a single recurrence. Paired isolates were not available for 14 patients, and a further eight isolates were missing from patients with more than one episode of recurrent disease. After these isolates were excluded, isolates from 116 patients with a total of 123 episodes of recurrent disease were available for typing. The time to recurrence for 123 episodes ranged from 15 to 3,757 days (10.3 years). The median time to recurrence was 317 days, with an interquartile range (IQR) of 129 to 930 days.

The PFGE banding patterns were identical for 90 of 123 episodes, including those episodes for five of the six patients with more than one recurrence. The PFGE banding patterns differed between isolate pairs in 33 episodes. This included an episode in one patient with two recurrent infections; the strain from the first recurrence had the same pattern as that of the strain from the primary infection, but the second recurrence was caused by an isolate with a different banding pattern. The
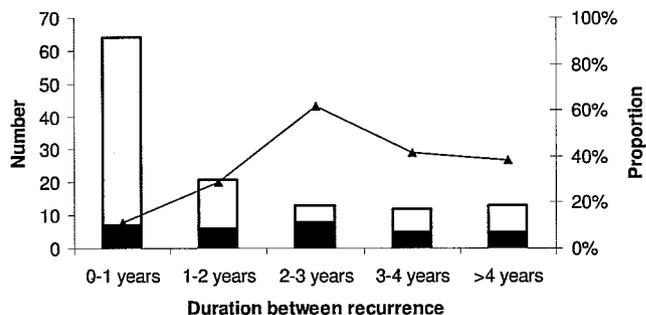

FIG. 1. Time to recurrent disease and proportion of cases due to reinfection. ■, reinfection; □, relapse; ▲, percent reinfection.

number of bands different between pairs ranged from 1 to 15 (median, 6 bands; IQR, 4 to 7 bands). Sequence analysis of one or both of *narK* and *gmhD* indicated that the isolates were different for 31 of the 33 episodes. Two isolate pairs that were one and four bands different, respectively, on PFGE were identical by full sequence typing of all seven loci. Thus, on the basis of combined typing, 92 episodes (75%) of recurrence represented relapsing disease and 31 episodes (25%) were due to reinfection with a new strain.

Of the six pairs classified as closely related by PFGE (up to three band differences) by the criteria of Tenover et al. (13), 5 (83%) were found to be different by sequenced-based typing. Of the 14 pairs classified as possibly related (four to six band differences), 13 (93%) were found to be different by sequence-based typing. All 13 pairs with greater than six band differences were different based on sequence-based typing.

The interval to recurrence differed for episodes due to relapse and reinfection; those caused by relapse had a median time of 228 days (range, 15 to 3,757 days; IQR, 99.5 to 608 days), while those due to reinfection had a median time of 823 days (range, 17 to 2,931 days; IQR 453 to 1,211 days) ($P = 0.0001$). The times to recurrence and the proportions due to reinfection are illustrated in Fig. 1. A total of 64 episodes (52%) occurred within 12 months of the primary infection. Recurrent disease was due to relapse in 57 of 64 episodes (89%) within the first year of the primary episode, in 15 of 21 episodes (71%) in the second year after the primary episode, and in 20 of 38 episodes (53%) more than 2 years after the primary episode.

There were only 12 instances where the colony morphology was completely different between the isolate pairs associated with the primary and the recurrent infection. Of these, only five (42%) represented relapses.

DISCUSSION

Previous studies of recurrent melioidosis have defined several clinical risk factors, including adherence to therapy, initial intravenous therapy, the severity of disease, and the duration of oral antibiotic therapy (2). Clinical trials have demonstrated higher recurrence rates in association with the use of doxycycline monotherapy (1), quinolone monotherapy (3), amoxicillin-clavulanate (11), and ciprofloxacin-azithromycin (4) in eradication therapy. Most of these studies have assumed that

recurrent melioidosis represents a relapse due to recrudescence of the original infecting isolate.

Previous studies appear to support this assumption. In an earlier study with our population, isolates from 23 of 25 patients with recurrent disease had identical or highly similar ribotype patterns, while those for two patients were different (6). A further study in Thailand reported different PFGE banding patterns for isolates from 4 of 35 patients with recurrent disease (8). In Australia, where recurrent disease was observed in 27 (13%) of patients over a follow-up period of 10 years, the majority of the cases (25 of 27) were due to relapse of the original infecting strain based on randomly amplified polymorphic DNA analysis or PFGE. In these patients, relapsed infection occurred between 1 and 27 months (mean, 8 months) after the primary presentation, whereas the two reinfections occurred after 14 months and 4.8 years (5). In a Malaysian study, four of five recurrences were due to relapse of the original infecting strain, based on ribotyping (14). Our contrasting findings are likely to reflect the long patient follow-up, with the longest recurrence occurring more than 10 years after the initial episode. The previous study with our Thai patient population had a shorter duration of follow-up, with only 6 of 25 episodes studied occurring after an interval of more than 12 months, of which 1 was defined as reinfection.

Use of different typing methods may also be important, since ribotyping may be less discriminatory than PFGE (10, 15). The criteria of Tenover et al. (13) have been widely adopted as the standard for the interpretation of DNA macrorestiction banding patterns in microepidemiological investigations; DNA macrorestriction patterns that differ by two to three bands are interpreted as being closely related, and differences of four to six bands are interpreted as possibly related (13). We used an unambiguous sequence-based typing system, MLST, to resolve the uncertainty for the DNA macrorestriction patterns that suggested that the strains were closely related. Use of these criteria at the level of "closely related" on PFGE alone would have led to the misclassification of five patients into the relapse group and one patient into the reinfection group. The predictive power of using colony morphology as a surrogate for strain typing was examined, but it was not found to be useful for this purpose.

In this study, we found that recurrent disease within 12 months was mostly due to relapse of the original infecting strain, consistent with previous results. However, the proportion of recurrent disease due to reinfection increased after this time; after 2 years, recurrence was equally due to reinfection as to relapse. The number of reinfections each year remained similar. We were unable to calculate a rate of reinfection, due to uncertainty in the number of patients being followed up at each point in time, but it is evident that this rate (~5 to 8 per 1,000 patients per year) is far greater than the annual incidence in the community (4.4 per 100,000 population) (12), illustrating the susceptibility of this group to infection with B. pseudomallei.

This study has important implications for future studies. After intervals exceeding 12 months, recurrent disease often represents reinfection rather than recrudescence of the original infecting isolate in our population. Studies of oral antibiotic eradication therapy for melioidosis that use recurrent disease as a marker of drug efficacy need to undertake bacterial typing to accurately distinguish relapse from reinfection, analogous to studies of antimalarials in areas where melioidosis is

highly endemic. Future typing studies should consider the use of sequence-based verification of the results for isolates that are different on PFGE. Patients with previous melioidosis form a group of individuals who are susceptible to subsequent infection; despite adequate oral eradication therapy, in areas of endemicity, reinfection following reexposure to environmental B. pseudomallei is more common than had previously been appreciated.

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