Journal of Microbiology, Immunology and Infection (2015) 48, 559–564

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ORIGINAL ARTICLE

Identification, epidemiological relatedness, and biofilm formation of clinical *Chryseobacterium indologenes* isolates from central Taiwan

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Received 2 September 2013; received in revised form 27 March 2014; accepted 1 April 2014
Available online 22 May 2014

KEYWORDS
Biofilm;
Central Taiwan;
Chryseobacterium indologenes;
Epidemiological relatedness;
Identification

Background: The clinical impact of *Chryseobacterium indologenes* infection is increasing; nevertheless, most studies had been conducted in northern Taiwan, but rarely in central Taiwan.
Methods: Using 16S rRNA gene sequencing, 34 isolates of *C. indologenes* were identified at the Central Region Hospital Alliance between 2007 and 2011. Vitek 2 and matrix-assisted laser desorption ionization–time-of-flight mass spectrometry (MALDI–TOF MS) methods were compared for the feasibility to identify this bacterium. Drug susceptibility test, biofilm formation, and pulsed-field gel electrophoresis (PFGE) were also performed.
Results: All isolates were collected from hospitalized patients with an average age of 70.8 ± 18.5years. The most prevalent sample was urine (50.0%), followed by sputum (32.4%). The accuracy rate of species-level identification reached 94.1% using the Vitek 2 method and 85.3% using the MALDI–TOF MS method. All of the isolates were resistant to gentamicin, amikacin, ceftriaxone, chloramphenicol, colistin, and imipenem, but completely susceptible to minocycline. While analyzing biofilm-forming ability, 38.2% (13/34) of *C. indologenes* isolates displayed a positive phenotype using the Luria–Bertani (LB) medium. However, 80.0% (4/5) of invasive isolates were biofilm producers. Based on PFGE analysis, several clusters were found, and the possible intrahospital spread of this bacterium in this area could not be excluded.

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http://dx.doi.org/10.1016/j.jmii.2014.04.004

Conclusion: Both Vitek 2 and MALDI—TOF MS methods showed good ability in the determination of *C. indologenes*. Among the examined drugs, minocycline was the most potent one. As many as 38.2% *C. indologenes* isolates showed biofilm-forming ability. PFGE analyses revealed the possible intrahospital transmission of this bacterium in central Taiwan.

Introduction

Chryseobacterium indologenes, previously classified under the *Flavobacterium* CDC group IIb,[1] is a rare human pathogen that is widely distributed in soil, plants, and water. The clinical importance of *C. indologenes* has increased in recent years after being identified as the causative agent of bacteriemia,[2] pneumonia,[3] indwelling device-associated infection,[4] peritonitis,[5] and ocular infection.[6] Bacteremia and pneumonia caused by this bacterium generally result in high mortality, which may partly be attributed to the inherent multidrug-resistant trait of *C. indologenes*.[7]

In clinical microbiology laboratories, conventional methods are generally used for the identification of glucose nonfermenting Gram-negative bacilli, including *C. indologenes*. However, the main drawback of conventional methods that detect the active metabolic processes in the bacteria is that these are time consuming. Some commercial kits, such as a Vitek 2 GN card, have been developed for saving of both time and labor.[8] When necessary, 16S rRNA gene sequencing, a molecular method, is recommended as the standard method for the identification of bacteria.[9] Nevertheless, complexity and high cost of the technique hamper its routine application. In recent years, matrix-assisted laser desorption ionization—time-of-flight mass spectrometry (MALDI—TOF MS) has widely been applied in the identification of many bacterial species, including glucose nonfermenting Gram-negative bacilli.[10,11] However, literature concerning the feasibility of this technique in the identification of *C. indologenes* is limited.

In addition, little was known about the virulence determinants of *Chryseobacterium* species.[12] Biofilm is a microbial community that attaches to a solid surface that is surrounded by extracellular polymeric substances produced by the microorganism(s).[13] The bacterial biofilm formed in the human body may lead to septicemia or more serious consequences.[13] Biofilm formation has been reported to be associated with infections caused by *C. indologenes* and a related species, *Elizabethkingia meningoseptica*.[14,15] However, to the best of our knowledge, no survey of the biofilm-forming ability of clinical *C. indologenes* isolates has been reported.

The first aim of this study was to compare Vitek 2 and MALDI—TOF MS methods for the identification of *C. indologenes* using 16S rRNA gene sequencing as the standard method. The potential biofilm-forming ability of this bacterium was then explored. In addition, antibiogram and pulsed-field gel electrophoresis (PFGE) pattern were investigated to provide more information regarding *C. indologenes* infection in central Taiwan.

Methods

Patients and bacterial isolates

C. indologenes isolates were collected by the Central Laboratory of the Central Region Hospital Alliance, including mainly Taichung Hospital, Fongyuan Hospital, Changhwa Hospital, and Nantou Hospital, from 2007 to 2011. All the patients' medical records were retrospectively studied.

16S rDNA identification

Genomic DNA extraction was performed using the genomic DNA purification kit (Biokit, Miaoli, Taiwan). A primer pair (5′-AGAGTTTGATCMTGGCTCAG-3′ and 5′-GGYTACCTTGTTACGACTT-3′) was used.[16] The subsequent polymerase chain reaction and DNA sequencing were performed as previously reported,[17] and then the DNA sequence data were BLASTed using the NCBI database.

MALDI—TOF MS

MALDI—TOF MS was performed using the Microflex LT system and analyzed by the Bruker Biotyper 3.0 software (Bruker Daltonics, Bremen, Germany). In brief, a score of <1.700 was interpreted as no identification, 1.700—1.999 indicated an identification to the genus level, 2.000—2.299 indicated a reliable identification of the genus and a probable species identification, and 2.300—3.000 represented a high probability of species identification.

Biochemical discrimination and antimicrobial susceptibility

Vitek 2 GN and AST cards (bioMérieux, Marcy-l'Etoile, France) were used for biochemical identification and drug susceptibility assay, respectively, according to the manufacturer's instruction. The minimum inhibitory concentrations (MICs) were interpreted according to the Clinical and Laboratory Standards Institute criteria for other non-Enterobacteriaceae.[18]

Biofilm analysis

The bacteria were inoculated on the brain heart infusion agar plates at 37°C for 18—20 hours, washed twice, and resuspended in different broths [LB, tryptic soy broth (TSB), or M9 minimal medium (M9)] to the turbidity of McFarland 0.5, and then diluted 100-fold with respective medium.

Subsequently, 125 μL of the bacterial suspension was transferred to a 96-well polyvinyl chloride microtiter plate and incubated at 37°C for 15 hours. Each well was washed thrice with sterile phosphate-buffered saline (pH 7.5) and stained with a 0.25% crystal violet solution. Triple reactions were performed for each isolate. Any isolate with absorption greater than twofold of the blank at OD_{570} was regarded as positive for biofilm formation.[19]

PFGE analysis

Plugs were prepared as previously described,[17] and the genomic DNA was digested with *Xho*I. The genomic DNA of *Salmonella enterica* serovar *Braenderup* H9812, digested with *Xba*I, was used as the molecular weight marker.[20] PFGE was performed using the CHEF-DR III system (Bio-Rad; Hercules, CA, USA). The gel was run at a field strength of 6.0 V/cm, at a reorientation angle of 120°, and from an initial switch time of 0.5 seconds to a final switch time of 26.4 seconds. The result was analyzed by GelComparII software (Applied Maths NV, St-Martens-Latem, Belgium). Dice similarity coefficients were calculated using unweighted pair group mean association.

Results

Characteristics of patients

The average age of the 34 patients was 70.8 ± 18.5 years, and only five (14.7%) patients were younger than 50 years. All were hospitalized patients and approximately half were female. Twenty-five patients had underlying diseases, including diabetes mellitus (n = 15, 44.1%), chronic renal failure (n = 9, 26.5%), and old stroke (n = 9, 26.5%; Table 1). The major sample was urine (50.0%), followed by sputum (32.4%).

Table 1 Demographic and clinical features of 34 patients with *Chryseobacterium indologenes* infection

Variables	No. (%)
Age (y)	70.8 ± 18.5
Female	18 (52.9)
Source	
Urine	17 (50.0)
Sputum	11 (32.4)
Blood	4 (11.8)
Cerebrospinal fluid, eye discharge	1 (2.9, each)
Hospital	
Fongyuan	14 (41.2)
Nantou	9 (26.5)
Taichung	6 (17.6)
Changhua	4 (11.8)
Penghu	1 (2.9)
Underlying disease	
Diabetes mellitus	15 (44.1)
Old stroke	9 (26.5)
Chronic renal failure	9 (26.5)
Hypertensive heart disease	7 (20.6)
Chronic obstructive pulmonary disease	5 (14.7)
Chronic respiratory failure	5 (14.7)
Cervical spondylosis, liver cirrhosis, phthisis, gastric cancer, lymphoma	1 (2.9, each)

Comparison of identification methods

Using 16S rRNA gene sequencing as the gold standard, 34 isolates were identified as *C. indologenes*, with sequence similarity ranging from 99.0% to 100% (data not shown). The identification results of the 34 isolates by the Vitek 2 and MALDI–TOF MS methods were compared (Table 2). By the Vitek 2 method, 32 isolates were identified as *C. indologenes* with acceptable discrimination (94.1%), whereas one isolate was identified as *C. indologenes* with unacceptable discrimination (2.9%) and one was regarded as *Chryseobacterium gleum* with acceptable discrimination (2.9%). Using MALDI–TOF MS, 29 isolates were correctly identified to the species level (85.3%), four to the genus level (11.8%), and one could not be identified (2.9%). MALDI–TOF MS failed to identify four isolates that were recognized using a Vitek 2 GN card (isolates 17, 24, 29, and 33; data not shown), but successfully distinguished one isolate that could not be classified by the Vitek 2 method (isolate 23; data not shown). However, one isolate could not be identified properly by both these methods (isolate 32).

Drug susceptibility

The 34 *C. indologenes* isolates were highly resistant to most drugs examined (Table 3). All the isolates were completely resistant to gentamicin, amikacin, ceftriaxone, chloramphenicol, colistin, and imipenem. Susceptibilities of the *C. indologenes* isolates to trimethoprim/sulfamethoxazole and ciprofloxacin were 52.9% (18/34) and 14.7% (5/34), respectively. The most potent drug examined was minocycline, to which isolates showed 100% susceptibility.

Biofilm formation

Of the *C. indologenes* isolates, 38.2% (13/34), 17.6% (6/34), and 0% (0/34) displayed biofilm-forming ability using the LB, TSB, and M9 media, respectively (Table 4). The biofilm-forming rate of *C. indologenes* isolates from sputum and urine was 45.5% (5/11) and 23.5% (4/17), respectively. As many as 80.0% (4/5) invasive isolates (4 from blood and 1 from cerebrospinal fluid) were biofilm producers.

PFGE analysis

Epidemiological relatedness of the 34 isolates from central Taiwan was determined by PFGE (Fig. 1). With a similarity coefficient of ≥75% as a cutoff value, four major clusters were found with seven, six, five, and four members, respectively, whereas the remaining 10 isolates were genetically diverse. In addition, two isolates (isolates 10 and 18) showed a smear of unresolvable DNA fragments after PFGE analyses (data not shown). Further examination of the hospital sources of these members among the four

Table 2 Comparison of Vitek 2 with Bruker Biotyper for *Chryseobacterium indologenes* identification

Bruker		Vitek 2		
Identification	Score[a]	*C. indologenes*		*Chryseobacterium gleum*
		Acceptable[b]	Unacceptable	Acceptable
C. indologenes	≥2.300	24	1	
	2.000–2.299	4		
Chryseobacterium sp.	1.700–1.999	3		1
No identification	<1.700	1		

[a] <1.700: no identification; 1.700–1.999: genus-level identification; 2.000–2.299: secure genus identification and probable species identification; 2.300–3.000: highly probable species identification.
[b] ≥85% probability.

Table 3 Drug susceptibility pattern of 34 isolates

Antimicrobial agent	MIC_{50} (μg/mL)	MIC_{90} (μg/mL)	Susceptibility breakpoint (μg/mL)	Susceptible (%)
Gentamicin	≥16	≥16	≤4	0
Amikacin	≥64	≥64	≤16	0
Ceftazidime	≥64	≥64	≤8	2.9
Ceftriaxone	≥64	≥64	≤8	0
Cefepime	≥64	≥64	≤8	2.9
Chloramphenicol	≥64	≥64	≤8	0
Ciprofloxacin	≥4	≥4	≤1	14.7
Colistin	≥16	≥16	≤2	0
Imipenem	≥16	≥16	≤4	0
Piperacillin	≥128	≥128	≤16	2.9
Piperacillin/tazobactam	≥128/4	≥128/4	≤16/4	2.9
Minocycline	≤1	≤1	≤4	100
TMP–SMZ	2/38	≥16/304	≤2/38	52.9

MIC = minimum inhibitory concentration; TMP–SMZ = trimethoprim/sulfamethoxazole.

major clusters demonstrated that each cluster contained multiple isolates from the same hospital (Fig. 1).

Discussion

Patients were all hospitalized and mostly older people with underlying disease(s) (73.5%). An equivalent or even higher prevalence of patients with various kinds of underlying diseases was observed in previous reports.[7,21] The two major samples in this study were urine and sputum. However, the most dominant sample previously reported is sputum.[7]

Table 4 Comparison of different media for the biofilm formation of *C. indologenes* isolates from different samples

Source	Biofilm formation (%)		
	TSB	LB	M9
Urine (n = 17)	3 (17.6)	4 (23.5)	0 (0.0)
Sputum (n = 11)	2 (18.1)	5 (45.5)	0 (0.0)
Blood (n = 4)	1 (25.0)	3 (75.0)	0 (0.0)
Cerebrospinal fluid (n = 1)	0 (0.0)	1 (100.0)	0 (0.0)
Eye discharge (n = 1)	0 (0.0)	0 (0.0)	0 (0.0)
Total (n = 34)	6 (17.6)	13 (38.2)	0 (0.0)

LB = Luria–Bertani; TSB = tryptic soy broth.

In this study, the correct rates for species-level identification by the Vitek 2 and MALDI–TOF MS methods were 94.1% and 85.3%, respectively. Even though these two methods had previously been employed for the identification of *C. indologenes*,[8,10,11] to the best of our knowledge, this was the first trial to compare these two methods focusing on this bacterium. Nevertheless, owing to the limited number of isolates used in this study, we tended not to conclude the priority of these two methods. Interestingly, isolate 32 was misidentified as *C. gleum* using Vitek 2 with a probability of 99.0% and recognized as *Chryseobacterium* species by MALDI–TOF MS method with a score of 1.771 (data not shown). It might imply the atypical nature of this isolate and emphasize the necessity of 16s rRNA gene sequencing as a standard method.

The multidrug-resistant trait of *C. indologenes* isolates from central Taiwan was as serious, if not more, as the isolates previously reported in other areas of Taiwan.[7,22] For example, studies conducted in northern[7] and southern[22] Taiwan revealed 87.4% and 100% susceptibilities to trimethoprim and sulfamethoxazole, respectively. However, only 52.9% susceptibility was observed in this study. Undoubtedly, minocycline, to which isolates showed 100% susceptibility in this survey and 92–100% in previous reports,[2,22] emerged as the most active drug against *C. indologenes* infection.

A higher rate of biofilm formation was found in sputum isolates than in urine isolates, which might reflect the

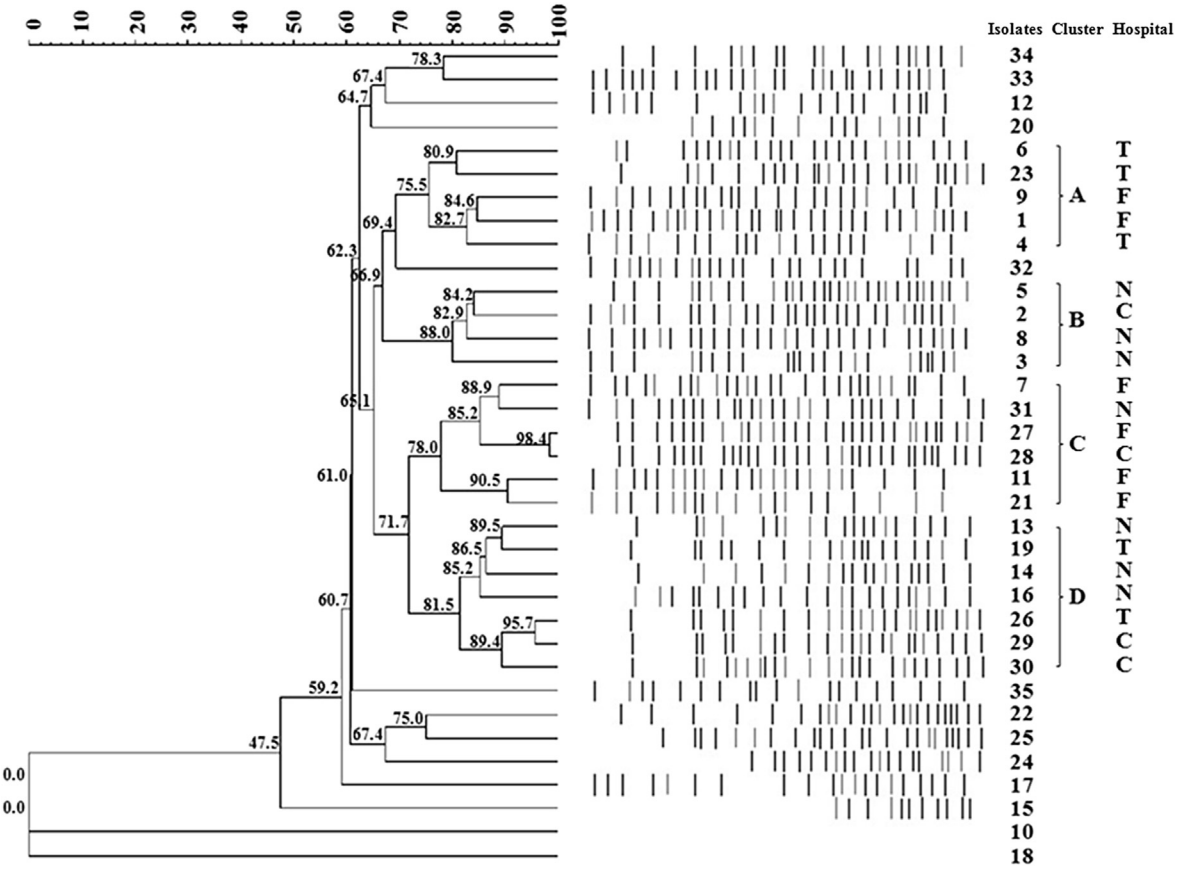

Figure 1. PFGE dendrogram of 34 *C. indologenes* isolates. The presenting cluster was grouped with a similarity coefficient of ≥75% as a cutoff value. C = Changhua Hospital; F = Fongyuan Hospital; N = Nantou Hospital; T = Taichung Hospital.

variation in colonization potential of *C. indologenes* at different sites. The observation of invasive isolates with a higher rate of biofilm formation led to the speculation of a pathogenetic role of biofilm in this bacterium. However, the small number ($N = 5$) of invasive isolates limited us from making a conclusion. LB showed the highest potential for biofilm formation (38.2%), whereas the M9 medium could not support this bacterium to form a biofilm. It is noteworthy that richer nutrient contents of a medium favor biofilm formation in *Hafnia alvei*.[23] However, our results revealed that the biofilm-forming ability of *C. indologenes* in the richer medium (TSB) was lower than that in the LB medium (Table 4). It was previously observed that *E. meningoseptica* isolates displayed a 100% biofilm-forming ability in the LB medium, and mortality was associated with an increased biofilm formation by this bacterium.[14] In an earlier study, *C. indologenes* causing multiple episodes of bacteremia by the same strain was thought to be related to biofilm formation.[4] Based on the diversity of the biofilm-forming potential between these two bacterial species, it was proposed that *C. indologenes* may be less virulent than *E. meningoseptica*. In addition, the minimum biofilm eradication concentration of imipenem toward *Pseudomonas aeruginosa* ATCC 27853 is 1000-fold higher than the minimum inhibitory concentration.[24] It is possible that the formation of biofilm by *C. indologenes* may also reduce its susceptibility to antimicrobial agents.

PFGE patterns revealed four major clusters with a similarity coefficient of ≥75% as a cutoff value. Each cluster contained multiple isolates from the same hospital (Fig. 1). Similarity cutoff values of 75–85% are generally applied for PFGE grouping of bacteria.[25,26] However, even with a cutoff value of 80% or 85%, we could observe related isolates (e.g., isolates 13, 14, and 16) that were from the same source (Fig. 1). In addition, these three isolates shared an identical susceptibility pattern (susceptible to minocycline, but resistant to all other drugs examined; data not shown). Thus, the possibility of an intrahospital spread of certain clone(s) in this area could not be excluded. Using random amplification of polymorphic DNA analysis, isolates recovered from different patients at a medical center in northern Taiwan within an 8-month period were proved to be clonally unrelated.[4] More recent research in northern Taiwan also demonstrated, using PFGE profiles, no cluster among *C. indologenes* bacteremic isolates.[7] Isolates from patients with cystic fibrosis in Italy were also found to be genetically independent.[27] Therefore, the finding of possible intrahospital transmission of *C. indologenes* in central Taiwan highlights the necessity of continuous monitoring in the future.

In this study, MALDI—TOF MS and Vitek 2 methods showed comparable ability in identifying *C. indologenes*. The 34 examined isolates were multidrug resistant, and 38.2% had biofilm-forming ability. It was noteworthy that both drug resistance and biofilm formation might increase the difficulty in treatment. In addition, epidemiological relatedness among some of the 34 isolates alerted the possibility of intrahospital spread in central Taiwan. Given the increasing clinical importance of *C. indologenes*, continuous surveillance of this bacterium in this area is recommended.

Conflicts of interest

All authors declare no conflicts of interest.

Acknowledgments

This study was supported partly by grants from the Fongyuan Hospital (101-009) and the Central Taiwan University of Science and Technology (FYH1011603-1). We are grateful to the staff of the Central Laboratory of Central Region Hospital Alliance who contributed to the collection of isolates.

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