Identification of *Inquilinus limosus* in Cystic Fibrosis: a first report in Italy

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SUMMARY

Cystic fibrosis is a genetic disorder associated with a polymicrobial lung infection where classical pathogens and newly identified bacteria may interact. *Inquilinus limosus* is an α-proteobacterium recently isolated in the airways of cystic fibrosis patient. We report the first case in Italy of *I.limosus* isolation from the sputum sample of a cystic fibrosis patient. The patient is a 20-years-old man with cystic fibrosis, regularly attending the Regional Care Center for Cystic Fibrosis at the Federico II University Hospital of Naples. Microbiological culture methods detected a mucoid gram negative bacillus in the patient's sputum sample. The isolate exhibited a distinct antimicrobial susceptibility profile with a high MIC for several drugs. The MALDI-TOF mass spectrometry analysis indicated the bacterium isolated as *I. limosus*, confirmed by 16s rDNA sequence analysis. The described clinical case demonstrates how the bacterial biodiversity in the airways of cystic fibrosis patients is still underestimated. Cystic fibrosis lung represents an ecological niche suitable for growth of a wide variety of unusual bacteria not commonly associated with human diseases, such as *I. limosus*. Therefore further studies are needed to evaluate the epidemiology and clinical implications of *I. limosus* in the physiopathology of cystic fibrosis lung infection.

KEY WORDS: Inquilinus limosus, Cystic fibrosis, Mucoid strain identification.

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INTRODUCTION

Cystic fibrosis (CF) lung infection is characterized by chronic infections caused by a variety of microorganisms. Over the past 20 years, the epidemiology of bacteria involved in acute infections in CF has become increasingly complex. Although *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Haemophilus influenzae* and *Burkholderia cepacia* complex, have been the most common pathogens in the lower airways of CF patients with improved survival, new pathogens, such as *Achromobacter xylosoxidans*, *Ralstonia pickettii*, and *Stenotrophomonas maltophilia* and other unusual bacteria such as *Acinetobacter* spp., *Bordetella* spp., *Moraxella* spp., *Comamonas* spp., *Rhizobium* spp., *Herbaspirillum* spp., and *Inquilinus limosus* have been detected in the last two decades (Bosch *et al.,* 2008; Coenye *et al.,* 2002; Fernández-Olmos *et al.,* 2012; Lopes *et al.,* 2012; Miller *et al.,* 2003; Raso *et al.,* 2008; Spilker *et al.,* 2008).

The standard laboratory methods available for isolation of bacteria from respiratory samples usually consist of selective media adapt-

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ed to the culture analysis of pathogens most frequently associated with CF (Rogers *et al.,* 2003). Correct identification of these pathogens is important as it underlies effective infection control measures and therapeutic intervention (Shreve *et al.,* 1999). However, several studies have shown that the identification of pathogens closely related to CF is far from straightforward (McMenamin *et al.,* 2000). Since conventional methods are not able to detect emerging species as potentially important pathogens in CF pulmonary infection only molecular biology techniques could categorise these isolates (Bittar *et al.,* 2008a). Recently, *Inquilinus limosus* has been increasingly found in CF specimens by molecular approaches as a novel microorganism (Bittar *et al.,* 2008a). This is a new multidrug-resistant species, belonging to the α proteobacteria; the genus *Azospirillum* is the most closely related bacteria (Coenye *et al.,* 2002). To date, 8 clinical cases have been described in Germany (Schmoldt *et al.,* 2006; Wellinghausen *et al.,* 2005), one case in the United States (Pitulle *et al.*, 1999), 5 cases in France (Chiron *et al.,* 2005), one case in the United Kingdom (Cooke *et al.,* 2007) and in Spain (Salvador-García *et al.,* 2013). Only one isolate of *Inquilinus* sp. has been recovered from blood samples of a patient without CF who had prosthetic valve endocarditis (Kiratisin *et al.,* 2006).

Literature data indicate that some CF patients showed clinical signs of acute respiratory exacerbation and spirometric deterioration (Chiron *et al.,* 2005; Wellinghausen *et al*., 2005, Hayes *et al.,* 2009). In addition a serological response with the detection of IgG antibodies against various I. *limosus* antigens (Schmoldt *et al.,* 2006) was reported, reflecting the pathogenic potential of this microorganism. Moreover, the multiresistant profile to antimicrobial agents, combined with its mucoid phenotype, may explain the ability of *I. limosus* to persist in the airways of CF patients.

In order to detect microbes in respiratory samples, including unusual non-fermenting Gram negative bacteria, all specimens were collected from patients regularly attending the Regional CF Care Center at the Federico II University Hospital of Naples from 2010 to 2013 and were processed according to standardized national guidelines (SIFC, http://www.sifc.it/).

A semiquantitative technique of the bacterial load, developed by Prof. N. Høiby (Koch *et al.,* 2000) was applied. All sputum samples were diluted with dithiothreitol (v/v) for 30 min at 37°C. For effective isolation of all potential pathogens 20 µl of sample were plated on different selective media: BD Sabouraud agar, with gentamicin and chloramphenicol, MacConkey agar (Simad), *Burkholderia cepacia* Selective agar (BCSA; Biomerieux), BD Trypticase Soy Agar with 5% Sheep Blood (TSA) and BD Chocolate Agar. All plates were incubated for 48 h at 37°C, and the BCSA agar was incubated for further 15 days at room temperature for the detection of unusual bacteria and *Mycobacterium abscessus*. *I. limosus* was recovered, for the first time in Italy, from the sputum sample of one patient on BCSA agar containing gentamicin [0.01 g/l], vancomycin [0.0025 g/l] and polymyxin [600,000 U/l] after 5 days of incubation.

CASE REPORT

The mucoid strain of *I. limosus* was isolated from the sputum sample of a 20-year-old male patient with CF. At 8 months the patient received a diagnosis of CF for gastrointestinal symptoms and upper airway infections, based on sweat chloride test over 60 mmol/liter and confirmed by genetic analysis. At isolation of *I. limosus* the patient did not show any biochemical alteration of blood samples, as well as lung function tests were within the normal range for age and gender. Less than one acute pulmonary exacerbation/year had been recorded in the last five years. Two episodes of acute pancreatitis were previously reported.

In the initial sputum sample, apart from ~ 10^6 CFU/ml of *H. influenzae*, 10^3 CFU/ml of a mucoid Gram-negative bacillus was isolated from BCSA agar after 5 days of incubation. The isolate was positive to oxidase and catalase reaction, grew well on BCSA Selective agar and TSA, but failed to grow on MacConkey agar. Subsequently the profile of antibiotic resistance was evaluated by Kirby-Bauer test and E-Test (Table 1), on Mueller-Hinton agar, incubated at 37°C for 48 h; the interpretation of results is based on the European Committee on Antimicrobial Susceptibility Testing (EUCAST)

TABLE 1 - *Antimicrobial susceptibility of Inquilinus limosus isolates.*

Antimicrobial agent Isolate A-1 (March 21, 2013) and Isolate A-2 (June 26, 2013)	*Kirby-Bauer*	*E-Test (MIC µg/ml)*
Amoxicillin/clavulanate	R	NS
Aztreonam	R	NS
Cefepime	R	R >256
Cefotaxime	R	NS
Ceftazidime	R	R >256
Ceftriaxone	R	NS
Piperacillin/tazobactam	R	R>256
Ciprofloxacin	S	S = 0.064
Gentamicin	R	NS
Amikacin	R	R>256
Netilmicin	R	NS
Imipenem	NS	S = 0.094
Meropenem	S	S = 0.094
Trimethoprim/ sulfamethoxazole	R	R >32
Colistin[a]	R	R >256

[a]The sensitivity tests done with disk showed no zone of inhibition. R, resistant; S, sensitive; NS, not screened.

guidelines. Surprisingly, the isolate showed a multi-resistant profile to several antimicrobial drugs, in particular, it showed a strong resistance to colistin, which is widely used for treatment for *P. aeruginosa* infection. Probably, the mucoid phenotype of the microorganism contributed to the colonization of the upper respiratory tract and the development of drug resistance (Bittar *et al*., 2008b). These characteristics have led us to hypothesize that *I. limosus* could be the pathogen, as recently reported (Chiron *et al*., 2005; Cooke *et al.,* 2007; Pitulle *et al.,* 1999; Salvador-García *et al.,* 2013; Schmoldt *et al*., 2006; Wellinghausen *et al.,* 2005). MALDI-TOF (Bruker) mass spectrometry analysis confirmed our hypothesis with a score value of 1.697. The final identification was performed by 16S rDNA sequence analysis (GeneBank database accession n. AY043375). Using multi-sequence alignment of all *I. limosus* 16S rDNA sequences available in the GeneBank database a pair of primers were designed: Il1r (5'-CACCCTCTCTTGGATTCAAGC-3') (Bittar *et al.,* 2008b) and Il16Sf (5'-CTTTGGCTAATACCGTATACG-3'), the obtained amplicon, 467 bp, was sequenced from CEINGE Advanced Biotechnologies s.c.ar.l., Naples, Italy.

The isolate showed a 99.9% sequence homology to the 16S rDNA sequence of the *I. limosus* type strain AU1979 by using the BLAST algorithm (Coenye *et al.,* 2002). The region of the 16S rDNA analyzed is highly specific for *I. limosus*, while it is not conserved in other Gram negative species, such as *E. coli* and *P. aeruginosa*.

A sputum sample collected three months later from the same patient confirmed isolation of *I. limosus*, which was identified in same way as the initial isolate.

The only difference was the bacterial load equal to 10^4 CFU/ml of *I. limosus* and 10^4 CFU/ml of *H. influenzae*. The patient was still in very good clinical conditions as indicated by lung function test (FEV1% predicted: 81) and no antibiotics therapeutic regimens were performed during this observation.

CONCLUSIONS

There is growing evidence in the literature that *I. limosus* can colonize the airways of CF patients, but its pathogenic potential and natural reservoir is still unclear. While its correlation with other non-fermentative rods suggests environmental sources, given its multiresistance to several antimicrobial drugs, this bacterium could be selected during the evolution of the disease. To our knowledge, isolation of *I. limosus* has not been described in Italy from clinical samples.

The identification of *I. limosus* is difficult due to its rather slow growth and inability to grow on MacConkey agar. Recovery of *I. limosus* can be improved by using selective media containing polymyxin B or colistin and ticarcillin, such as BCSA agar, and prolonged incubation at room temperature. This failure to grow on MacConkey agar, positive oxidase reaction, and typical antimicrobial susceptibility profile (Table 1) should raise suspicion.

I. limosus shows a distinct antimicrobial susceptibility profile with high MICs for cefepime, ceftazidime, piperacillin/tazobactam, amikacin, trimethoprim/sulfamethoxazole and colistin. *I. limosus* may be effectively protected from the action of antimicrobial agents by high mucus production. In our report the conclusive identification of *I. limosus* was achieved using

MALDI-TOF mass spectrometry and confirmed by 16S rRNA gene sequencing.

The described clinical case does not seem to support the pathogenic role of this microorganism as the patient showed a good lung function and clinical stability during the observation period. Therefore further studies are needed to evaluate the epidemiology and clinical implications of *I. limosus*.

Although the role of this bacterium in the physiopathology of the pulmonary infections is not known, the description of new bacterial species represents the first step to understand their involvement in the physiopathology of CF lung infection.

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