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DNA Typing of Cytological Samples for Retrospective Identification of an Early Case of Panton-Valentine Leucocidin-Positive, Community-Associated Methicillin-Resistant *Staphylococcus aureus* Pneumonia▽

P. L. Ho,[1]* M. P. Wong,[2] Eileen L. Lai,[1] K. H. Chan,[1] and Susan S. Chiu[3]

Departments of Microbiology,[1] Pathology,[2] and Paediatrics and Adolescent Medicine,[3] Queen Mary Hospital, University of Hong Kong, Hong Kong Special Administrative Region, China

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This paper describes a fatal case of culture-confirmed, community-associated methicillin-resistant *Staphylococcus aureus* (MRSA) pneumonia in an 8-month-old child in Hong Kong in 2001. Stored cytological materials prepared from the pleural fluid were retrieved for molecular analysis. The result indicates the presence of a Panton-Valentine leucocidin-positive, *spa* type 019 MRSA.

CASE REPORT

The patient was an 8-month-old infant. The mother was a housewife (Filipino ethnicity), while the father was a public service officer (Indian ethnicity). In March 2001, the patient presented to our hospital with a 3-day history of cough, malaise, and poor appetite. He had had fever since the night before and shortness of breath in the morning of the admission date. The patient had no significant medical history. There was no history of recent travel. A physical examination revealed a temperature of 39°C, a heart rate of 130 beats/min, a respiratory rate of 60 respirations/min, and an oxygen saturation of 93% by pulse oximetry. Chest examination revealed bilateral crepitations and a mild wheeze.

The complete blood picture revealed leukopenia (white cell count, 1.8×10^9/liter), thrombocytopenia (platelet count, 148×10^9/liter), and anemia (hemoglobin, 8.4 g/dl). The serum creatinine level and liver biochemistry were normal. A chest X-ray showed extensive, bilateral lung consolidation in all zones and air bronchogram and cystic changes in several areas. The patient was resuscitated, and empirical intravenous amoxicillin-clavulanate and cloxacillin were started. Initially, he was put on a 50% oxygen Venturi mask for respiratory support. Nonetheless, the patient's condition deteriorated rapidly, and he was transferred to the intensive care unit for intubation and mechanical ventilation. Subsequently, the antimicrobial therapy was changed to intravenous vancomycin and cefotaxime. The clinical course was further complicated by bilateral pneumothorax, requiring insertion of chest drains. Despite maximal support, the patient succumbed 26 h after hospitalization.

Culture of nasal and throat swabs revealed parainfluenza virus type 3. Tracheal aspirate fluid and pleural fluids from both lungs revealed methicillin-resistant *Staphylococcus aureus* (MRSA) that was sensitive to vancomycin, erythromycin, clindamycin, fusidic acid, gentamicin, and cotrimoxazole. The *S. aureus* was identified by Gram staining, colony morphology, and latex agglutination (Slidex Staph Plus, bioMerieux, France) and tube coagulase test results. Antimicrobial susceptibility testing was performed by the disc diffusion method in accordance with the CLSI (3). An oxacillin disc was used for phenotypic detection of methicillin resistance. The pleural fluid was grossly blood stained and had total cell counts of 196×10^6/liter. Microscopic examination showed a moderate number of macrophages and lymphocytes with a few polymorphs. A blood culture performed after the initiation of antibiotics showed no growth after 7 days of incubation. An examination of the lung at autopsy showed extensive suppurative pneumonia, and staining showed the presence of colonies of gram-positive cocci.

Since the MRSA isolates were not stored, we attempted to investigate the molecular characteristics of the organism 6 years later by using the stored cytological material prepared from the pleural fluid and paraffin-embedded lung tissue blocks. All the samples were kept at room temperature inside storage boxes. The following target-specific primer pairs were used: forward, ATG AAG TGA ACT GGA AAA CTC A, and reverse, TGT ATT GGA TAG CAA AAG CAA TG, for *lukS-lukF* genes (114 bp) (this study); MECA P4 and MECA P7 for *mecA* (162 bp) (3); and Sa442-1 and Sa442-2 for an *S. aureus*-specific target, *sau* (108 bp) (8). In addition, the primers SpaF1 and SpaR1 were used to amplify the polymorphic X region of protein A (3). The NucliSENS easyMAG (bioMérieux, France) nucleic acid extraction kit was used for lysis, extraction, and purification of nucleic acid. The kit utilizes silica-coated paramagnetic beads for the concentration of nucleic acid and the removal of enzyme inhibitors. A recent evaluation indicated that it may extract nucleic acid more efficiently from clinical samples than the Qiagen kit does (7). The results showed the presence of *mecA*, *sau*, *spa*, and *lukS-lukF* genes in the samples. Sequencing of the *spa* amplicons indicated the presence of *spa* type 019 nucleic acid. Tests of two lung tissue samples yielded identical results. All PCR and sequencing analyses were conducted twice on two indepen-

* Corresponding author. Mailing address: Dept. of Microbiology, University Pathology Building, Queen Mary Hospital, Pokfulam Road, Hong Kong Special Administrative Region, China. Phone: 852-28554892. Fax: 852-28551241. E-mail: plho@hkucc.hku.hk.

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dently obtained DNA extracts, with identical results. DNA extraction, PCR amplification, and all subsequent manipulations were conducted in the virology or histopathology section of the University of Hong Kong. The sections were located in buildings different from the bacteriology section of the laboratory. No culture or other molecular assays of *S. aureus* occurred in those sections. Precautions recommended for the handling of ancient DNA samples were adopted to prevent contamination (10). Attempts to confirm the sequence type were unsuccessful because the longer loci (>450 bp) could not be amplified.

Panton-Valentine leucocidin (PVL)-producing, community-associated MRSA (CA-MRSA) is an emerging cause of fulminant community-acquired pneumonia worldwide (2, 6, 9). The disease tends to occur in healthy children and young adults with no underlying medical conditions but with earlier or concurrent respiratory viral infections and to exhibit a fulminant clinical course with rapid patient deterioration following pulmonary necrosis, hemorrhage, and respiratory failure (9). In Hong Kong, little is know about PVL-positive CA-MRSA pneumonia. Therefore, we reviewed the database for a retrospective cohort of children that had been constructed earlier for the estimation of pneumonia burden (4). An MRSA case was considered to be community associated if it was isolated from an outpatient or within 48 h of the patient's hospitalization. Exclusion criteria included a history of hospitalization for illness (except birth), surgery, or dialysis in the previous year and (2) the presence of indwelling catheters or other medical devices. Among 890 children hospitalized in the Queen Mary Hospital from January 2000 to December 2006 because of clinical pneumonia, one case was found to have a history consistent with CA-MRSA pneumonia. Here, we describe the clinical details of the patient and the application of DNA typing to stored cytological materials for inferring the *S. aureus* as PVL-positive CA-MRSA. The finding indicates that CA-MRSA occurred in this locality several years before its first recognition in 2004 (5).

As far as we know, this is the first study which utilizes stored cytological specimens for retrospective ascertainment of CA-MRSA. The present case isolate shared several features common to the predominant ST30-HKU100 CA-MRSA clone in this locality, including a nonmultiresistant susceptibility pattern, the presence of PVL, and the protein A polymorphic sequence of t019 (3). In Hong Kong, the ST30-HKU100 clone was recognized to be overrepresented among persons of non-Chinese ethnicity (12). The parental ethnicity of the present case is in agreement with this observation. For antimicrobial treatment, some groups have advocated the early use of protein synthesis inhibitors (e.g., linezolid or clindamycin) once PVL-positive CA-MRSA pneumonia is suspected (9, 11), because nafcillin has been shown to upregulate PVL toxin production (11). Nonetheless, it is still unknown if PVL plays a key role in the pathogenesis of necrotizing *S. aureus* pneumonia. According to Labandeira-Rey et al. (6), PVL toxin is a major virulence factor in lung infections but that study was criticized for the use of a laboratory strain (RN6390). In another study which used clinical CA-MRSA strains (LAC and MW2, representing the USA300 and USA400 clones, respectively), Hla toxin but not PVL was found to be essential for staphylococcal pneumonia (1). In conclusion, this case demonstrates the potential utility of stored cytological material for retrospective study of CA-MRSA epidemiology.

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