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Epidemiology of borderline oxacillin-resistant *Staphylococcus aureus* in pediatric cystic fibrosis.

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**Keywords:** methicillin resistant *Staphylococcus aureus* (MRSA), *mecA*, pulsed-field gel electrophoresis.

**Abbreviated title.** BORSA in pediatric cystic fibrosis
Summary

A single-centre retrospective study was undertaken in children with cystic fibrosis (CF) to evaluate 1) risk factors for acquisition; 2) molecular epidemiology; and 3) impact on disease progression of borderline oxacillin-resistant *Staphylococcus aureus* (BORSA) versus *mecA*-positive methicillin-resistant *Staphylococcus aureus* (MRSA). The study comprised of 1) identification of all children with at least one respiratory specimen positive for either BORSA or MRSA during the study period; 2) compilation of relevant clinical and epidemiological data from 12-month period leading up to first positive (index) culture; 3) microbiological and molecular characterisation of index isolates and 4) measurement of subsequent clinical outcome. Thirty-eight children were identified with at least one positive isolate; 4 were excluded due to insufficient clinical or laboratory data. Eighteen children (53%) grew BORSA in their index culture. Children who acquired BORSA only (n=16) were more likely to have had prior MSSA colonization (p<0.0001). Usage of oral cephalxin (p<0.01) and inhaled tobramycin (p<0.03) prior to index culture was significantly and independently associated with acquisition of BORSA. The majority of BORSA isolates were hyper β-lactamase producers and susceptible to a greater range of antibiotics. Strain relatedness was not evident within the BORSA group. There was no difference in disease progression between the two groups. This is the first study to demonstrate that a significant proportion of *S. aureus* isolates with methicillin resistance in the CF population are BORSA that lack *mecA*. Antibiotic pressure may lead to the development of BORSA in CF patients. Prospective studies are needed to assess its clinical impact.
Introduction

Methicillin-resistant *Staphylococcus aureus* (MRSA) has emerged as an important respiratory pathogen in the cystic fibrosis (CF) population. The US CF registry reported a significant rise in MRSA infection among CF patients, from 2.1% in 1996 to 21.2% in 2007. MRSA infection has been associated with accelerated deterioration in pulmonary function, increased hospitalization, impaired growth, increased antibiotic usage and increased mortality in CF patients.

According to the Clinical Laboratory Standards Institute (CLSI), methicillin resistance in *S. aureus* is defined as having an oxacillin minimum inhibitory concentration (MIC) > 4 µg/ml. The primary mechanism of resistance for MRSA is the production of an altered penicillin binding protein (PBP2a) with reduced affinity for methicillin that is encoded by the *mecA* gene. We have identified a group of CF patients infected with borderline resistant *S. aureus* (BORSA) isolates that are characterized by oxacillin MICs at or just above the susceptibility breakpoint of 4 µg/ml and lack the *mecA* gene. The implications of isolating BORSA in a respiratory culture from CF patients have not previously been explored. The aim of this study was therefore to compare the risk factors for acquisition, the molecular epidemiology and subsequent clinical impact on disease progression of BORSA infection in comparison with *mecA*-positive MRSA infection in pediatric CF patients.
68 **Materials and Methods**

69

**Study design and patient population**

This was a single-centre retrospective comparative study of pediatric CF patients whose care was primarily based at the CF clinic at the Hospital for Sick Children, Toronto between 1992 and 2007. The clinic currently cares for approximately 280 children up to the age of eighteen. Respiratory samples (expectorated sputum or oropharyngeal swabs) were sent from all children attending the clinic to the microbiology laboratory for culture at every 3-month visit and during acute pulmonary exacerbations (APEs). Patients with one or more respiratory specimens positive for *S. aureus* with methicillin resistance (either BORSA or MRSA) were included in the analysis. Cases with three or more specimens positive for BORSA or MRSA during the study period were considered persistently colonized. The study was approved by the Hospital for Sick Children’s Research Ethics Board.

**Data collection**

Patients with BORSA or MRSA isolated from their respiratory specimens were identified using the microbiology database, and cross-referenced with the CF clinical database. Date of the first BORSA or MRSA-positive respiratory culture (index culture) for each patient was identified. The medical and laboratory records for each patient were examined using a standardized data collection form. Baseline patient characteristics were assessed at the date of index culture, and risk factors for acquisition of BORSA or MRSA infection were assessed over the twelve-month period prior to the index culture. Impact
on clinical progression was assessed by examination of the time period from index
culture until discharge from the clinic or until December 31st 2008, whichever date was
later. Data collected included information on patients’ demographics, pulmonary function
tests (PFTs), respiratory cultures, hospital admissions, acute pulmonary exacerbations
and antibiotic use. An acute pulmonary exacerbation was defined as a change in
pulmonary status considered by the attending physician to require antibiotic treatment,
either oral or intravenous. An “antibiotic day” was defined as one day for each antibiotic
used, for example a 14-day course of two antibiotics was defined as 28 “antibiotic days”.
The purpose of this distinction was to capture more complex antibiotic regimens that
might be employed to treat MRSA infected patients. Patients were classified as being on
oral cephalexin if they were prescribed cephalexin at the time of index culture of at least
one month’s duration (in all instances, this occurred in the absence of signs or symptoms
of a pulmonary exacerbation). Patients were classified as being on inhaled tobramycin if
they were prescribed inhaled tobramycin at the time of index culture of at least one
month’s duration (for previous P. aeruginosa infection). Patients were classified as being
on oral ciprofloxacin if they were prescribed at least one course of ciprofloxacin (in all
instances, this occurred in the presence of signs or symptoms of a pulmonary
exacerbation). Patients were categorized as co-colonized with other CF pathogens using
previously defined criteria \(^8\). Only patients with at least 6 months of follow-up after the
index culture were included in the analysis of outcome measures.
Microbiology

Specimen Processing

Respiratory specimens from CF patients were screened for MRSA using media containing 2 µg/ml of oxacillin: an in-house mannitol salt agar (MRSA agar base, Acumedia, Lansing, MI, and 1% D-mannitol, Difco, Detroit, MI ) supplemented with 1% thymidine and 2 µg/ml of oxacillin (both from Sigma, St. Louis, MO) was used from 1992 to 2001, and Oxacillin Resistance Screening Agar Base (ORSAB, Oxoid, Nepean, Ontario, Canada) was used from 2002 onward. Plates were incubated at 35°C aerobically and observed for the presence of growth at 24 hours and at 48 hours. Suspicious colonies were identified as *S. aureus* by Gram stain, catalase, slide coagulase, and tube coagulase tests.

Antimicrobial Susceptibility

Antimicrobial susceptibility of *S. aureus* isolates was tested by a combination of oxacillin 6µg/ml screen plate, disk diffusion and an automated system (Vitek, bio-Merieux, St. Laurent, Quebec, from 1992 to Nov 2002, and the BD Automated Phoenix System, BD Diagnostic Systems, Sparks, MD from Nov 2002 onward). Inducible resistance to clindamycin was detected by disk diffusion using the D-zone test. In addition, each strain was examined for the presence of PBP2a by latex agglutination (PBP2’ test kit, Oxoid, Hants, UK). Organisms that were negative for PBP2a by latex agglutination but grew on the oxacillin screen plate were sent to the reference laboratory (Central Provincial Health Laboratory, Ontario) for minimum inhibitory concentration (MIC) by agar dilution and polymerase chain reaction (PCR) for the detection of the
meca gene. An isolate was deemed as BORSA if the oxacillin MIC was $\geq 4 \mu g/ml$, PBP2a was absent and the meca PCR was negative\(^7\). An isolate was deemed as MRSA if the oxacillin MIC was $\geq 4 \mu g/ml$, the latex agglutination for PBP2a was positive and/or the meca PCR was positive\(^6\).

**β-Lactamase Production**

All BORSAs were tested for the production of β-lactamase as a potential mechanism of oxacillin resistance by disk diffusion with ampicillin (10 µg) and amoxicillin-clavulanic (20 µg/10 µg) disks as previously described (13). An isolate was deemed as hyper β-lactamase producing if the ampicillin zone size was resistant and the amoxicillin-clavulanic zone size was susceptible, suggesting borderline resistance was secondary to large amounts of β-lactamase\(^9\) (13).

**Molecular Characterization**

The meca and nuc gene were co-amplified with a multiplex real time PCR as described previously\(^{10}\). The presence of Panton-Valentine leukocidin was examined by amplification of the lukF-PV and lukS-PV genes using primer and protocols previously described\(^{11}\). Pulsed-field gel electrophoresis (PFGE) was conducted using SmaI as per manufacturer protocol (Bio-rad Laboratories, Hercules, CA) on all newly identified MRSA strains. Electrophoretically-generated DNA profiles were analyzed using the criteria proposed by Tenover et al to determine strain relatedness\(^{12}\).
Descriptive statistics, frequency distributions and percentages were calculated for the outcome variables and other covariates of interest. Normality assumption test was carried out for the continuous variables using the Kolmogorov-Smirnov test. Student’s t-test or Kruskal-Wallis test (if normality assumption failed) was conducted for continuous variables within the two groups. The Chi-square or Fisher’s exact test, as appropriate, was carried out for the dichotomous variables to assess the association with the two groups. To analyze independent risk factors, a multivariate analysis using a logistic regression model was applied. A p-value of <0.05 was considered statistically significant. The statistical programme SAS (version 9.1; SAS Institute Inc., Cary, NC) was used.
Results

Patient population

Between 1992 and 2007, 38 patients had at least one respiratory specimen positive for either BORSA or MRSA. Of these, three were excluded from our analysis because their care was not primarily based at our hospital, thus we had no clinical data for these patients. In addition, mecA PCR could not be performed on one isolate retrospectively therefore 34 patients were included in the study. Of these 34 patients, two patients were initially colonized with mecA-negative and subsequently mecA-positive strains and were excluded from the final statistical analysis as it was unclear how to categorize them in terms of patient characteristics and outcomes. Ultimately 16 patients with mecA positive MRSA and 16 with mecA negative BORSA on the index culture were compared for baseline characteristics. For the longitudinal assessment, 15 patients in each group were compared since one patient in each group did not have follow-up data for at least 6 months.

Patient characteristics and risk factors for BORSA and MRSA

The two groups were similar in terms of baseline characteristics (Table 1). There was no difference in pulmonary function or rate of decline of pulmonary function in the year prior to the index culture. In addition, there was no difference in the number of acute pulmonary exacerbations, hospital admissions or overall antibiotic use for the treatment of acute pulmonary exacerbations. A significantly greater proportion of BORSA infected patients were colonized with MSSA in the year prior (94% vs 25%, p<0.0001). Likewise,
a higher proportion of BORSA infected patients received oral antibiotics (cephalexin) (75% vs 25%, p=0.005).

Mean duration of therapy among those patients on cephalexin was 337 days (range 30-365 days) in the BORSA group and 311 days (range 150-365 days) in the MRSA group; cephalexin treatment was instituted not for pulmonary exacerbation but as treatment for MSSA identified on routine sputum cultures. Furthermore, a significantly greater number of BORSA infected patients had received a course of oral ciprofloxacin (31% vs 0%, p=0.043) or were on inhaled antibiotics (tobramycin) (88% vs 44%, p=0.009) in the year prior to the index culture than patients with MRSA. No inter-group difference was detected in any of the other co-colonizing respiratory pathogens. Patients with BORSA strains had significantly more clinic visits in the year prior to first isolate (n=7.94) than patients with meca-positive strains (n=4.69, p=0.049) although this was not a significant risk factor in the logistic regression model.

A logistic regression model was applied to determine the independence of epidemiological and clinical variables that were correlated with BORSA infection. Colonization with MSSA and use of oral cephalexin were significantly associated as 68% of patients with MSSA were treated with cephalexin and 81% of patients treated with cephalexin were colonized with MSSA (p<0.05). The use of oral cephalexin (regression coefficient (β)=1.15, SD=0.47, p value=0.015) and inhaled tobramycin (regression coefficient (β)=1.16, SD=0.53, p value=0.028) in the year prior to index culture were significantly and independently associated with BORSA infection.

Microbiological characteristics of BORSA and MRSA isolates
The microbiological characteristics of the BORSA isolates are illustrated in Table 2. Fourteen of the 16 isolates had oxacillin MICs at or just above the breakpoint (4 to 8 µg/ml) but 2 isolates had MICs of 16 µg/ml. All of the BORSA isolates were negative for PBP2a by latex agglutination and were mecA negative by PCR. Twelve of the 16 BORSA isolates were hyper β-lactamase producers. This was shown by resistance to ampicillin by disk diffusion that was reverted to susceptible with the addition of clavulanic acid, a β-lactamase inhibitor. The remaining 4 isolates had ampicillin zone size that were unchanged by addition of clavulin by disk diffusion.

In comparison to the MRSA isolates, a significantly greater proportion of the BORSA isolates were susceptible to both erythromycin (81% vs 13%, p < 0.0001) and clindamycin (94% vs 31%, p<0.001). None of the BORSA isolates were D-test positive but 5 (45%) of the MRSA isolates were D-test positive (p=0.043). All of the BORSA and MRSA isolates were susceptible to trimethoprim-sulphamethoxazole. None of the BORSA isolates tested produced PVL. Three of the 16 MRSA isolates were identified as USA300 epitypes, and produced PVL.

Relatedness of BORSA and MRSA isolates

Pulsed field gel electrophoresis (PFGE) revealed that none of the BORSA strains were related by the Tenover criteria 13 (Figure 1). Within the MRSA group, we found four separate instances of strain relatedness during the course of the study: a pair of siblings shared an indistinguishable strain, four patients shared possibly related strains, two patients shared a closely related strain with a third patient sharing a possibly related
strain and, finally, three patients (including a pair of siblings) shared an indistinguishable strain.

Clinical outcomes of patients

To compare the clinical impact of BORSA versus MRSA infection on disease progression, we assessed 99.7 patient-years of follow-up after the index culture with a comparable mean duration of follow up of 3.37 years in the MRSA group and 3.27 years in the BORSA group (p=0.507) (Table 3). Patients infected with MRSA were more likely to have persistent infection at the end of follow up than patients with BORSA infection (80% vs 53%) but this was not statistically significant (p=0.121). The annual decline in percent predicted forced expiratory volume in 1 second (FEV₁), was greater among the MRSA infected patients (5.8%) than those infected with BORSA (2.8%) although this difference was not significant (p = 0.77). Neither was there a significant difference in the number of acute pulmonary exacerbations, hospital admissions, inpatient days or clinic visits. One patient in the MRSA group died, and another progressed to lung transplant. Three patients in the BORSA group had a lung transplant during the study period.
Discussion

This is the first study to show that a significant proportion (50%) of *S. aureus* isolates with methicillin resistance infecting CF patients are BORSA which lack the meca gene, the main genetic determinant of methicillin resistance in *S. aureus*\(^7\). The present study demonstrates that antibiotic use in CF patients colonized with MSSA is a risk factor specifically for infection with BORSA. Previous studies have shown that ciprofloxacin and cephalosporin use is a risk factor for acquisition of MRSA in CF patients\(^14\) but, to our knowledge, no other study has examined the differential risk factors for BORSA compared to MRSA acquisition.

Transmission of traditional MRSA between patients\(^15,16\), within the community\(^17\) and even within households\(^18\) has been well described in the CF population. Evidence of person to person transmission of meca-positive MRSA was also present in this study with shared strains among CF patients and siblings. The mechanism of resistance is relevant to how CF patients acquire MRSA as the meca gene is absent in its entirety from MSSA\(^19,7\). It is believed to have originated from another staphylococcus species, such as *Staphylococcus sciuri*, and been transposed into MSSA\(^20\). Although horizontal transfer of meca between a *S. epidermidis* and MSSA isolate within the same patient has been described\(^21\), transmission of meca-positive MRSA isolates between individuals is likely more common as there are a well defined number of meca-positive MRSA clones worldwide\(^22\).

Our data would suggest that CF patients may acquire meca-negative BORSA isolates in a different way. In contrast to MRSA isolates, BORSA isolates had unique
PFGE patterns and appeared not to be shared between patients in our study. CF patients colonized with MSSA and exposed to oral cephalexin or inhaled tobramycin (which can act synergistically with β-lactam antibiotics against *S. aureus*) were at significantly increased risk of acquiring BORSA infection. This suggests that BORSA isolates may arise from endogenous MSSA isolates that develop other mechanisms of resistance to methicillin in response to antibiotic pressure. Previous studies have shown that *S. aureus* strains with borderline susceptibility to oxacillin have a significant drop in the penicillin MICs to the susceptible range with the addition of a β-lactamase inhibitor\(^2\)\(^3\). This predicts the response of oxacillin MICs to the addition of a β-lactamase inhibitor and suggests that a large amount of β-lactamase production is the mechanism of resistance to oxacillin in the majority of BORSA, as observed in 12 of 16 isolates in our study.

However, in the 4 remaining BORSA strains where the ampicillin zone size did not change significantly with the addition of clavulanic acid, the mechanism of methicillin resistance is unknown. In the aforementioned study, the authors described *S. aureus* strains with intermediate MICs to oxacillin that remained the same in the presence of clavulanic acid. The authors suggested that oxacillin resistance was due to factors other than β-lactamase inactivation\(^2\)\(^3\). It is possible that it is due to a modification of “normal” penicillin-binding proteins (PBP) such as PBP 1, PBP 2 and PBP 4 as was previously shown in clinical BORSA isolates by Tomasz et al\(^2\)\(^4\). Exposure of susceptible staphylococci to increasing concentrations of β-lactam antibiotics in the laboratory is known to cause PBP alterations associated with increased MICs\(^2\)\(^5\). In this study, persistent antibiotic pressure may have similarly modified PBPs in endogenous MSSA isolates colonizing CF patients and resulting in the development of *mecA*-negative, non-
hyper β-lactamase producing BORSA isolates. Thus, in the prevention of the emergence of BORSA in CF patients, other infection control measures, such as patient segregation, may not be as important as the judicious use of antibiotics.

In our study, the majority of patients treated with oral cephalexin were colonized with MSSA. Although there were no signs and symptoms of a pulmonary exacerbation at the time, we are limited in a retrospective study to the information that is recorded in the medical charts. Their antibiotic treatment was thus likely driven by their MSSA positive respiratory cultures and/or more subtle symptoms. Primary prophylaxis for *S. aureus* (the treatment of all newly diagnosed CF patients with antistaphylococcal agents irrespective of respiratory culture results) is not routinely practiced in North America due to concerns of *P. aeruginosa* acquisition. A systematic review of prophylactic antibiotics in cystic fibrosis, however, suggests that *P. aeruginosa* acquisition in this context is associated less frequently with narrow spectrum anti-staphylococcal antibiotics such as flucloxacillin compared with broader spectrum anti-staphylococcal antibiotics such as cephalexin. In our study, although patients treated with oral cephalexin were not receiving prophylaxis against *S. aureus*, they were receiving prolonged courses of cephalexin for MSSA detected on routine culture of the respiratory tract. This prolonged cephalexin use was identified as an independent risk factor for the acquisition of BORSA isolates and occurred recently, primarily in the last 5 years of the study.

The clinical significance of BORSA infection in CF patients is unknown. Our study lacked a sufficient sample size and is not powered to clearly delineate the clinical impact of BORSA infection. Previous studies have reported accelerated deterioration in pulmonary function, increased hospitalization, impaired growth, increased antibiotic use, and other outcomes. Further research is needed to fully understand the impact of BORSA on CF patients.
usage \(^4\) and increased mortality \(^5\) in CF patients infected with MRSA. However, these studies are limited by a lack of information on the laboratory methodology of identifying MRSA and thus it is not known to what degree these effects are attributable to BORSA or MRSA.

Detection of BORSA isolates may also influence the choice of antibiotics in the treatment of these infections in the setting of a pulmonary exacerbation. In our study, BORSA isolates were more susceptible to erythromycin and clindamycin than the MRSA isolates. This finding is not surprising as the \textit{mecA} gene is located on a mobile genetic element (the Staphylococcal Cassette Chromosome \textit{mec}: SCC\textit{mec}) which can contain additional drug resistance genes \(^22\). Although earlier studies suggested that infections due to \textit{mecA} negative BORSA isolates could be treated with \(\beta\)-lactam antibiotics \(^29,30\), more recent cases report failure of cloxacillin and even vancomycin in the treatment of endocarditis caused by BORSA, depending on the mechanism of methicillin resistance \(^31,32\). This has implications for how respiratory specimens from CF patients should be screened for MRSA. Some of the newer MRSA selective media such as chromogenic media for MRSA that use cefoxitin as the selective agent will not detect \textit{mecA}-negative BORSA isolates as we have observed in our own clinical laboratory. Thus these isolates could be missed from CF respiratory specimens unless full antimicrobial susceptibilities were performed on all \textit{S. aureus} isolates. Hence, CF patients could potentially be treated with suboptimal antibiotics during a pulmonary exacerbation.

There were several limitations to this study. It is unclear whether CF patients infected with BORSA isolates behave more like CF patients infected with MSSA or CF patients infected with MRSA. Future studies assessing the clinical impact of BORSA in
CF should compare BORSA, MRSA and MSSA colonized patients in a longitudinal fashion, controlling for potential confounding factors and include more extensive PFGE comparisons to determine the evolution of BORSA. In addition, the small number of patients limited the number of variables that could be included in the logistic regression model of risk factors. This study was also not powered to detect differences in clinical outcomes between the groups. It was a single centre study and may not reflect findings in other CF populations. Finally, in a quarter of patients with mecA-negative BORSA, the mechanism of resistance to methicillin remains unknown.

In conclusion, CF patients with mecA-positive MRSA most commonly acquire their infection through person to person transmission whereas BORSA is likely preferentially selected out from endogenous MSSA in CF patients due to persistent antibiotic pressure. Prospective studies are needed to examine the prevalence, clinical impact and appropriate management of BORSA in the CF population, as well as the underlying mechanism of resistance.
Acknowledgements

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References


Figure legends

Figure 1.

A. PFGE of Smal restriction fragments of representative MRSA isolates (lanes 2-6).

Strains in lanes 3, 4 are indistinguishable and strains in lanes 2, 6 are closely related.

Lanes 1,7: lambda ladder.

B. Representative BORSA isolates (lanes 1-5) that are unrelated. Lane 6: lambda ladder.
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Introduction

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According to the Clinical Laboratory Standards Institute (CLSI), methicillin resistance in *S. aureus* is defined as having an oxacillin minimum inhibitory concentration (MIC) > 4 µg/ml. The primary mechanism of resistance for MRSA is the production of an altered penicillin binding protein (PBP2a) with reduced affinity for methicillin that is encoded by the *meca* gene. We have identified a group of CF patients infected with borderline resistant *S. aureus* (BORSA) isolates that are characterized by oxacillin MICs at or just above the susceptibility breakpoint of 4 µg/ml and lack the *meca* gene. The implications of isolating BORSA in a respiratory culture from CF patients have not previously been explored. The aim of this study was therefore to compare the risk factors for acquisition, the molecular epidemiology and subsequent clinical impact on disease progression of BORSA infection in comparison with *meca*-positive MRSA infection in pediatric CF patients.
Materials and Methods

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Data collection

Patients with BORSA or MRSA isolated from their respiratory specimens were identified using the microbiology database, and cross-referenced with the CF clinical database. Date of the first BORSA or MRSA-positive respiratory culture (index culture) for each patient was identified. The medical and laboratory records for each patient were examined using a standardized data collection form. Baseline patient characteristics were assessed at the date of index culture, and risk factors for acquisition of BORSA or MRSA infection were assessed over the twelve-month period prior to the index culture. Impact
on clinical progression was assessed by examination of the time period from index culture until discharge from the clinic or until December 31\textsuperscript{st} 2008, whichever date was later. Data collected included information on patients’ demographics, pulmonary function tests (PFTs), respiratory cultures, hospital admissions, acute pulmonary exacerbations and antibiotic use. An acute pulmonary exacerbation was defined as a change in pulmonary status considered by the attending physician to require antibiotic treatment, either oral or intravenous. An “antibiotic day” was defined as one day for each antibiotic used, for example a 14-day course of two antibiotics was defined as 28 “antibiotic days”. The purpose of this distinction was to capture more complex antibiotic regimens that might be employed to treat MRSA infected patients. Patients were classified as being on oral cephalexin if they were prescribed cephalexin at the time of index culture of at least one month’s duration (in all instances, this occurred in the absence of signs or symptoms of a pulmonary exacerbation). Patients were classified as being on inhaled tobramycin if they were prescribed inhaled tobramycin at the time of index culture of at least one month’s duration (for previous \textit{P. aeruginosa} infection). Patients were classified as being on oral ciprofloxacin if they were prescribed at least one course of ciprofloxacin (in all instances, this occurred in the presence of signs or symptoms of a pulmonary exacerbation). Patients were categorized as co-colonized with other CF pathogens using previously defined criteria \textsuperscript{8}. Only patients with at least 6 months of follow-up after the index culture were included in the analysis of outcome measures.
Microbiology

Specimen Processing

Respiratory specimens from CF patients were screened for MRSA using media containing 2 µg/ml of oxacillin: an in-house mannitol salt agar (MRSA agar base, Acumedia, Lansing, MI, and 1% D-mannitol, Difco, Detroit, MI) supplemented with 1% thymidine and 2 µg/ml of oxacillin (both from Sigma, St. Louis, MO) was used from 1992 to 2001, and Oxacillin Resistance Screening Agar Base (ORSAB, Oxoid, Nepean, Ontario, Canada) was used from 2002 onward. Plates were incubated at 35°C aerobically and observed for the presence of growth at 24 hours and at 48 hours. Suspicious colonies were identified as *S. aureus* by Gram stain, catalase, slide coagulase, and tube coagulase tests.

Antimicrobial Susceptibility

Antimicrobial susceptibility of *S. aureus* isolates was tested by a combination of oxacillin 6µg/ml screen plate, disk diffusion and an automated system (Vitek, bio-Merieux, St. Laurent, Quebec, from 1992 to Nov 2002, and the BD Automated Phoenix System, BD Diagnostic Systems, Sparks, MD from Nov 2002 onward). Inducible resistance to clindamycin was detected by disk diffusion using the D-zone test. In addition, each strain was examined for the presence of PBP2a by latex agglutination (PBP2’ test kit, Oxoid, Hants, UK). Organisms that were negative for PBP2a by latex agglutination but grew on the oxacillin screen plate were sent to the reference laboratory (Central Provincial Health Laboratory, Ontario) for minimum inhibitory concentration (MIC) by agar dilution and polymerase chain reaction (PCR) for the detection of the
meca gene. An isolate was deemed as BORSA if the oxacillin MIC was \( \geq 4 \mu g/ml \), PBP2a was absent and the meca PCR was negative\(^7\). An isolate was deemed as MRSA if the oxacillin MIC was \( \geq 4\mu g/ml \), the latex agglutination for PBP2a was positive and/or the meca PCR was positive\(^6\).

### β-Lactamase Production

All BORSAs were tested for the production of β-lactamase as a potential mechanism of oxacillin resistance by disk diffusion with ampicillin (10 µg) and amoxicillin-clavulinic (20 µg/10 µg) disks as previously described (13). An isolate was deemed as hyper β-lactamase producing if the ampicillin zone size was resistant and the amoxicillin-clavulanic zone size was susceptible, suggesting borderline resistance was secondary to large amounts of β-lactamase\(^9\) (13).

### Molecular Characterization

The meca and nuc gene were co-amplified with a multiplex real time PCR as described previously\(^10\). The presence of Panton-Valentine leukocidin was examined by amplification of the lukF-PV and lukS-PV genes using primer and protocols previously described\(^11\). Pulsed-field gel electrophoresis (PFGE) was conducted using SmaI as per manufacturer protocol (Bio-rad Laboratories, Hercules, CA) on all newly identified MRSA strains. Electrophoretically-generated DNA profiles were analyzed using the criteria proposed by Tenover et al to determine strain relatedness\(^12\).
Statistical analysis

Descriptive statistics, frequency distributions and percentages were calculated for the outcome variables and other covariates of interest. Normality assumption test was carried out for the continuous variables using the Kolmogorov-Smirnov test. Student’s t-test or Kruskal-Wallis test (if normality assumption failed) was conducted for continuous variables within the two groups. The Chi-square or Fisher’s exact test, as appropriate, was carried out for the dichotomous variables to assess the association with the two groups. To analyze independent risk factors, a multivariate analysis using a logistic regression model was applied. A p-value of <0.05 was considered statistically significant. The statistical programme SAS (version 9.1; SAS Institute Inc., Cary, NC) was used.
Results

Patient population

Between 1992 and 2007, 38 patients had at least one respiratory specimen positive for either BORSA or MRSA. Of these, three were excluded from our analysis because their care was not primarily based at our hospital, thus we had no clinical data for these patients. In addition, meca PCR could not be performed on one isolate retrospectively therefore 34 patients were included in the study. Of these 34 patients, two patients were initially colonized with meca-negative and subsequently meca-positive strains and were excluded from the final statistical analysis as it was unclear how to categorize them in terms of patient characteristics and outcomes. Ultimately 16 patients with meca positive MRSA and 16 with meca negative BORSA on the index culture were compared for baseline characteristics. For the longitudinal assessment, 15 patients in each group were compared since one patient in each group did not have follow-up data for at least 6 months.

Patient characteristics and risk factors for BORSA and MRSA

The two groups were similar in terms of baseline characteristics (Table 1). There was no difference in pulmonary function or rate of decline of pulmonary function in the year prior to the index culture. In addition, there was no difference in the number of acute pulmonary exacerbations, hospital admissions or overall antibiotic use for the treatment of acute pulmonary exacerbations. A significantly greater proportion of BORSA infected patients were colonized with MSSA in the year prior (94% vs 25%, p<0.0001). Likewise,
a higher proportion of BORSA infected patients received oral antibiotics (cephalexin) (75% vs 25%, p=0.005).

Mean duration of therapy among those patients on cephalaxin was 337 days (range 30-365 days) in the BORSA group and 311 days (range 150-365 days) in the MRSA group; cephalaxin treatment was instituted not for pulmonary exacerbation but as treatment for MSSA identified on routine sputum cultures. Furthermore, a significantly greater number of BORSA infected patients had received a course of oral ciprofloxacin (31% vs 0%, p=0.043) or were on inhaled antibiotics (tobramycin) (88% vs 44%, p=0.009) in the year prior to the index culture than patients with MRSA. No inter-group difference was detected in any of the other co-colonizing respiratory pathogens. Patients with BORSA strains had significantly more clinic visits in the year prior to first isolate (n=7.94) than patients with meca-positive strains (n=4.69, p=0.049) although this was not a significant risk factor in the logistic regression model.

A logistic regression model was applied to determine the independence of epidemiological and clinical variables that were correlated with BORSA infection. Colonization with MSSA and use of oral cephalaxin were significantly associated as 68% of patients with MSSA were treated with cephalaxin and 81% of patients treated with cephalaxin were colonized with MSSA (p<0.05). The use of oral cephalaxin (regression coefficient (\(\beta\))=1.15, SD=0.47, p value=0.015) and inhaled tobramycin (regression coefficient (\(\beta\))=1.16, SD=0.53, p value=0.028) in the year prior to index culture were significantly and independently associated with BORSA infection.

**Microbiological characteristics of BORSA and MRSA isolates**
The microbiological characteristics of the BORSA isolates are illustrated in Table 2. Fourteen of the 16 isolates had oxacillin MICs at or just above the breakpoint (4 to 8 µg/ml) but 2 isolates had MICs of 16 µg/ml. All of the BORSA isolates were negative for PBP2a by latex agglutination and were mecA negative by PCR. Twelve of the 16 BORSA isolates were hyper β-lactamase producers. This was shown by resistance to ampicillin by disk diffusion that was reverted to susceptible with the addition of clavulanic acid, a β-lactamase inhibitor. The remaining 4 isolates had ampicillin zone size that were unchanged by addition of clavulin by disk diffusion.

In comparison to the MRSA isolates, a significantly greater proportion of the BORSA isolates were susceptible to both erythromycin (81% vs 13%, p <0.0001) and clindamycin (94% vs 31%, p<0.001). None of the BORSA isolates were D-test positive but 5 (45%) of the MRSA isolates were D-test positive (p=0.043). All of the BORSA and MRSA isolates were susceptible to trimethoprim-sulphamethoxazole. None of the BORSA isolates tested produced PVL. Three of the 16 MRSA isolates were identified as USA300 epitypes, and produced PVL.

Relatedness of BORSA and MRSA isolates

Pulsed field gel electrophoresis (PFGE) revealed that none of the BORSA strains were related by the Tenover criteria 13 (Figure 1). Within the MRSA group, we found four separate instances of strain relatedness during the course of the study: a pair of siblings shared an indistinguishable strain, four patients shared possibly related strains, two patients shared a closely related strain with a third patient sharing a possibly related
strain and, finally, three patients (including a pair of siblings) shared an indistinguishable strain.

Clinical outcomes of patients

To compare the clinical impact of BORSA versus MRSA infection on disease progression, we assessed 99.7 patient-years of follow-up after the index culture with a comparable mean duration of follow up of 3.37 years in the MRSA group and 3.27 years in the BORSA group (p=0.507) (Table 3). Patients infected with MRSA were more likely to have persistent infection at the end of follow up than patients with BORSA infection (80% vs 53%) but this was not statistically significant (p=0.121). The annual decline in percent predicted forced expiratory volume in 1 second (FEV$_1$), was greater among the MRSA infected patients (5.8%) than those infected with BORSA (2.8%) although this difference was not significant (p = 0.77). Neither was there a significant difference in the number of acute pulmonary exacerbations, hospital admissions, inpatient days or clinic visits. One patient in the MRSA group died, and another progressed to lung transplant. Three patients in the BORSA group had a lung transplant during the study period.
Discussion

This is the first study to show that a significant proportion (50%) of S. aureus isolates with methicillin resistance infecting CF patients are BORSA which lack the \textit{mecA} gene, the main genetic determinant of methicillin resistance in S. aureus \textsuperscript{7}. The present study demonstrates that antibiotic use in CF patients colonized with MSSA is a risk factor specifically for infection with BORSA. Previous studies have shown that ciprofloxacin and cephalosporin use is a risk factor for acquisition of MRSA in CF patients \textsuperscript{14} but, to our knowledge, no other study has examined the differential risk factors for BORSA compared to MRSA acquisition.

Transmission of traditional MRSA between patients \textsuperscript{15,16}, within the community \textsuperscript{17} and even within households \textsuperscript{18} has been well described in the CF population. Evidence of person to person transmission of \textit{mecA}-positive MRSA was also present in this study with shared strains among CF patients and siblings. The mechanism of resistance is relevant to how CF patients acquire MRSA as the \textit{mecA} gene is absent in its entirety from MSSA \textsuperscript{19,7}. It is believed to have originated from another staphylococcus species, such as \textit{Staphylococcus sciuri}, and been transposed into MSSA \textsuperscript{20}. Although horizontal transfer of \textit{mecA} between a \textit{S. epidermidis} and MSSA isolate within the same patient has been described \textsuperscript{21}, transmission of \textit{mecA}-positive MRSA isolates between individuals is likely more common as there are a well defined number of \textit{mecA}-positive MRSA clones worldwide \textsuperscript{22}.

Our data would suggest that CF patients may acquire \textit{mecA}-negative BORSA isolates in a different way. In contrast to MRSA isolates, BORSA isolates had unique
PFGE patterns and appeared not to be shared between patients in our study. CF patients colonized with MSSA and exposed to oral cephalexin or inhaled tobramycin (which can act synergistically with β-lactam antibiotics against *S. aureus*) were at significantly increased risk of acquiring BORSA infection. This suggests that BORSA isolates may arise from endogenous MSSA isolates that develop other mechanisms of resistance to methicillin in response to antibiotic pressure. Previous studies have shown that *S. aureus* strains with borderline susceptibility to oxacillin have a significant drop in the penicillin MICs to the susceptible range with the addition of a β-lactamase inhibitor. This predicts the response of oxacillin MICs to the addition of a β-lactamase inhibitor and suggests that a large amount of β-lactamase production is the mechanism of resistance to oxacillin in the majority of BORSA, as observed in 12 of 16 isolates in our study.

However, in the 4 remaining BORSA strains where the ampicillin zone size did not change significantly with the addition of clavulanic acid, the mechanism of methicillin resistance is unknown. In the aforementioned study, the authors described *S. aureus* strains with intermediate MICs to oxacillin that remained the same in the presence of clavulanic acid. The authors suggested that oxacillin resistance was due to factors other than β-lactamase inactivation. It is possible that it is due to a modification of “normal” penicillin-binding proteins (PBPs) such as PBP 1, PBP 2 and PBP 4 as was previously shown in clinical BORSA isolates by Tomasz et al. Exposure of susceptible staphylococci to increasing concentrations of β-lactam antibiotics in the laboratory is known to cause PBP alterations associated with increased MICs. In this study, persistent antibiotic pressure may have similarly modified PBPs in endogenous MSSA isolates colonizing CF patients and resulting in the development of *mecA*-negative, non-
hyper β-lactamase producing BORSA isolates. Thus, in the prevention of the emergence of BORSA in CF patients, other infection control measures, such as patient segregation, may not be as important as the judicious use of antibiotics.

In our study, the majority of patients treated with oral cephalexin were colonized with MSSA. Although there were no signs and symptoms of a pulmonary exacerbation at the time, we are limited in a retrospective study to the information that is recorded in the medical charts. Their antibiotic treatment was thus likely driven by their MSSA positive respiratory cultures and/or more subtle symptoms. Primary prophylaxis for *S. aureus* (the treatment of all newly diagnosed CF patients with antistaphylococcal agents irrespective of respiratory culture results) is not routinely practiced in North America due to concerns of *P. aeruginosa* acquisition. A systematic review of prophylactic antibiotics in cystic fibrosis, however, suggests that *P. aeruginosa* acquisition in this context is associated less frequently with narrow spectrum anti-staphylococcal antibiotics such as flucloxacillin compared with broader spectrum anti-staphylococcal antibiotics such as cephalexin. In our study, although patients treated with oral cephalexin were not receiving prophylaxis against *S. aureus*, they were receiving prolonged courses of cephalexin for MSSA detected on routine culture of the respiratory tract. This prolonged cephalexin use was identified as an independent risk factor for the acquisition of BORSA isolates and occurred recently, primarily in the last 5 years of the study.

The clinical significance of BORSA infection in CF patients is unknown. Our study lacked a sufficient sample size and is not powered to clearly delineate the clinical impact of BORSA infection. Previous studies have reported accelerated deterioration in pulmonary function, increased hospitalization, impaired growth, increased antibiotic use, and other complications.
usage \(^4\) and increased mortality \(^5\) in CF patients infected with MRSA. However, these studies are limited by a lack of information on the laboratory methodology of identifying MRSA and thus it is not known to what degree these effects are attributable to BORSA or MRSA.

Detection of BORSA isolates may also influence the choice of antibiotics in the treatment of these infections in the setting of a pulmonary exacerbation. In our study, BORSA isolates were more susceptible to erythromycin and clindamycin than the MRSA isolates. This finding is not surprising as the \(mecA\) gene is located on a mobile genetic element (the Staphylococcal Cassette Chromosome \(mec\): SCC\(mec\)) which can contain additional drug resistance genes \(^22\). Although earlier studies suggested that infections due to \(mecA\) negative BORSA isolates could be treated with \(\beta\)-lactam antibiotics \(^29,30\), more recent cases report failure of cloxacillin and even vancomycin in the treatment of endocarditis caused by BORSA, depending on the mechanism of methicillin resistance \(^31,32\). This has implications for how respiratory specimens from CF patients should be screened for MRSA. Some of the newer MRSA selective media such as chromogenic media for MRSA that use cefoxitin as the selective agent will not detect \(mecA\)-negative BORSA isolates as we have observed in our own clinical laboratory. Thus these isolates could be missed from CF respiratory specimens unless full antimicrobial susceptibilities were performed on all \(S.\ aureus\) isolates. Hence, CF patients could potentially be treated with suboptimal antibiotics during a pulmonary exacerbation.

There were several limitations to this study. It is unclear whether CF patients infected with BORSA isolates behave more like CF patients infected with MSSA or CF patients infected with MRSA. Future studies assessing the clinical impact of BORSA in
CF should compare BORSA, MRSA and MSSA colonized patients in a longitudinal fashion, controlling for potential confounding factors and include more extensive PFGE comparisons to determine the evolution of BORSA. In addition, the small number of patients limited the number of variables that could be included in the logistic regression model of risk factors. This study was also not powered to detect differences in clinical outcomes between the groups. It was a single centre study and may not reflect findings in other CF populations. Finally, in a quarter of patients with mecA-negative BORSA, the mechanism of resistance to methicillin remains unknown.

In conclusion, CF patients with mecA-positive MRSA most commonly acquire their infection through person to person transmission whereas BORSA is likely preferentially selected out from endogenous MSSA in CF patients due to persistent antibiotic pressure. Prospective studies are needed to examine the prevalence, clinical impact and appropriate management of BORSA in the CF population, as well as the underlying mechanism of resistance.
Acknowledgements

The authors wish to acknowledge the assistance of Dr ML Mulvey at the National Reference Laboratory for his assistance in performing multiplex PCR for the MRSA isolates, Dana Kovach, Division of Microbiology in performing the pulsed field gel electrophoresis and Derek Stephens of the Child Health Evaluative Service for his assistance in the data analysis.
References


Figure legends

Figure 1.

A. PFGE of Smal restriction fragments of representative MRSA isolates (lanes 2-6).

Strains in lanes 3, 4 are indistinguishable and strains in lanes 2, 6 are closely related.

Lanes 1,7: lambda ladder.

B. Representative BORSA isolates (lanes 1-5) that are unrelated. Lane 6: lambda ladder.
Table 1. Characteristics of patients with MRSA and BORSA

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<td>No. (%) male</td>
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<td>Mean no. (range) of IV antibiotic days</td>
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Abbreviations: BMI – body mass index, MSSA- methicillin sensitive *Staphylococcus aureus*, FEV₁- Forcible expiratory volume in 1 second.
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<td>Mean no. (range) of IV antibiotic days</td>
<td>6.1 (0-70)</td>
<td>14.2 (0-130)</td>
<td>&gt;0.5</td>
</tr>
<tr>
<td>Mean no. (range) of clinic visits</td>
<td>4.7 (3-10)</td>
<td>7.9 (3-35)</td>
<td>0.049</td>
</tr>
</tbody>
</table>

Abbreviations: BMI – body mass index, MSSA- methicillin sensitive *Staphylococcus aureus*, FEV<sub>1</sub>- Forced expiratory volume in 1 second.
Table 2. Microbiological characteristics of BORSA isolates

<table>
<thead>
<tr>
<th>Isolate</th>
<th>Oxacillin MIC (µg/ml)</th>
<th>PBP2a latex agglutination</th>
<th>mecA PCR</th>
<th>AMP/AMC zone size (mm)†</th>
<th>Hyper β-lactamase production</th>
<th>ERY</th>
<th>CLI</th>
<th>SXT</th>
</tr>
</thead>
<tbody>
<tr>
<td>SA 002</td>
<td>8</td>
<td>neg</td>
<td>neg</td>
<td>13/23</td>
<td>Yes</td>
<td>S</td>
<td>S</td>
<td>S</td>
</tr>
<tr>
<td>SA 003</td>
<td>16</td>
<td>neg</td>
<td>neg</td>
<td>12/26</td>
<td>Yes</td>
<td>I</td>
<td>S*</td>
<td>S</td>
</tr>
<tr>
<td>SA 004</td>
<td>4</td>
<td>neg</td>
<td>neg</td>
<td>38/40</td>
<td>No</td>
<td>S</td>
<td>S</td>
<td>S</td>
</tr>
<tr>
<td>SA 006</td>
<td>8</td>
<td>neg</td>
<td>neg</td>
<td>35/36</td>
<td>No</td>
<td>S</td>
<td>S</td>
<td>S</td>
</tr>
<tr>
<td>SA 011</td>
<td>8</td>
<td>neg</td>
<td>neg</td>
<td>18/29</td>
<td>Yes</td>
<td>S</td>
<td>S</td>
<td>S</td>
</tr>
<tr>
<td>SA 016</td>
<td>4</td>
<td>neg</td>
<td>neg</td>
<td>15/26</td>
<td>Yes</td>
<td>S</td>
<td>S</td>
<td>S</td>
</tr>
<tr>
<td>SA 018</td>
<td>8</td>
<td>neg</td>
<td>neg</td>
<td>14/28</td>
<td>Yes</td>
<td>S</td>
<td>S</td>
<td>S</td>
</tr>
<tr>
<td>SA 020</td>
<td>4</td>
<td>neg</td>
<td>neg</td>
<td>17/28</td>
<td>Yes</td>
<td>S</td>
<td>S</td>
<td>S</td>
</tr>
<tr>
<td>SA 021</td>
<td>4</td>
<td>neg</td>
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<td>16/26</td>
<td>Yes</td>
<td>S</td>
<td>S</td>
<td>S</td>
</tr>
<tr>
<td>SA 022</td>
<td>8</td>
<td>neg</td>
<td>neg</td>
<td>30/29</td>
<td>No</td>
<td>S</td>
<td>S</td>
<td>S</td>
</tr>
<tr>
<td>SA 023</td>
<td>8</td>
<td>neg</td>
<td>neg</td>
<td>16/21</td>
<td>Yes</td>
<td>R</td>
<td>S*</td>
<td>S</td>
</tr>
<tr>
<td>SA 029</td>
<td>16</td>
<td>neg</td>
<td>neg</td>
<td>10/27</td>
<td>Yes</td>
<td>S</td>
<td>S</td>
<td>S</td>
</tr>
<tr>
<td>SA 034</td>
<td>8</td>
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<td>12/21</td>
<td>Yes</td>
<td>S</td>
<td>S</td>
<td>S</td>
</tr>
<tr>
<td>SA 035</td>
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<td>29/31</td>
<td>No</td>
<td>S</td>
<td>S</td>
<td>S</td>
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<tr>
<td>SA 036</td>
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<td>neg</td>
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<td>16/23</td>
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<td>S</td>
<td>S</td>
<td>S</td>
</tr>
<tr>
<td>SA 038</td>
<td>8</td>
<td>neg</td>
<td>neg</td>
<td>11/24</td>
<td>Yes</td>
<td>R</td>
<td>S*</td>
<td>S</td>
</tr>
</tbody>
</table>

†Ampicillin (AMP) susceptible zone size ≥ 29 mm, resistant ≤ 28 mm
Amoxicillin clavulanic acid (AMC) susceptible zone size ≥ 20 mm, resistant ≤ 19 mm

*D-test negative, AMP-ampicillin, AMC-amoxicillin clavulanic acid
ERY-erythromycin, CLI-clindamycin, SXT-trimethoprim-sulphamethoxazole
S-susceptible I-intermediate R-resistant
Table 3. Clinical outcomes of patients with MRSA and BORSA

<table>
<thead>
<tr>
<th>Clinical outcomes</th>
<th>MRSA (n = 15)</th>
<th>BORSA (n = 15)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean duration (range) of follow up in years</td>
<td>3.37 (0.76-14.8)</td>
<td>3.27 (0.66-5.57)</td>
<td>0.507</td>
</tr>
<tr>
<td>Mean annual change (range) in % predicted FEV1</td>
<td>-5.8 (-33.1 to +3.2)</td>
<td>-2.8 (-10.2 to +7.7)</td>
<td>0.770</td>
</tr>
<tr>
<td>Mean no. (range) of exacerbations per year</td>
<td>1.8 (0-5.3)</td>
<td>2.4 (0.3-7.6)</td>
<td>0.443</td>
</tr>
<tr>
<td>Mean no. (range) of hospital admissions per year</td>
<td>0.9 (0-6.6)</td>
<td>1.3 (0-4.7)</td>
<td>0.242</td>
</tr>
<tr>
<td>Mean no. (range) of inpatient days per year</td>
<td>11.5 (0-109.2)</td>
<td>16.1 (0-91.7)</td>
<td>0.226</td>
</tr>
<tr>
<td>Mean no. (range) of IV antibiotic days per year</td>
<td>26.1 (0-224.1)</td>
<td>33.3 (0-200.6)</td>
<td>0.361</td>
</tr>
<tr>
<td>Mean no. (range) of clinic visits per year</td>
<td>7.7 (3.7-37.4)</td>
<td>11.7 (2.8-80.9)</td>
<td>0.272</td>
</tr>
<tr>
<td>No. (%) of patients with persistent infection</td>
<td>12 (80)</td>
<td>8 (53.3)</td>
<td>0.121</td>
</tr>
</tbody>
</table>