A Caenorhabditis elegans host model correlates with invasive disease caused by Staphylococcus aureus recovered during an outbreak in neonatal intensive care

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BACKGROUND: Caenorhabditis elegans has previously been used as a host model to determine the virulence of clinical methicillin-resistant Staphylococcus aureus isolates. In the present study, methicillin-susceptible S aureus (MSSA) strains associated with an outbreak in a neonatal intensive care unit (NICU) were investigated using the C elegans model.

METHODS: Two distinct outbreak clones, MSSA type-C and MSSA type-G, were identified by pulsed-field gel electrophoresis in a MSSA outbreak during a seven-month period in the NICU of the Sunnybrook Health Sciences Centre (Toronto, Ontario). MSSA type-C was associated with severe infection, while type-G was associated with less invasive disease. Four representative type-C isolates, three type-G and three infant-colonized isolates unrelated to the outbreak, were sent to Calgary (Alberta), for the double-blinded virulence tests in the C elegans host model and for further molecular characterization.

RESULTS: The invasive outbreak strains (type-C) demonstrated highly nematocidal activity, the noninvasive outbreak strains (type-G) an intermediate virulence, and the outbreak-unrelated colonization isolates demonstrated avirulence or low virulence in the C elegans model, with mean killing rates of 93.0%, 61.0% and 14.4% by day 9, respectively, for these three group strains. Different group MSSA strains had their own unique genetic profiles and virulence gene profiles, but all isolates within the same group (type-C or type-G) shared identical genetic characteristics and virulence gene patterns.

CONCLUSIONS: The present blinded evaluation demonstrated that methicillin-resistant S aureus (MRSA) have been frequently identified as causes of outbreaks of infection in NICUs (2).

We previously used the nematode, Caenorhabditis elegans, as a host model to determine the virulence of clinical MRSA isolates and demonstrated that the virulence of MRSA to C elegans correlated well with the isolation of MRSA from clinically relevant invasive anatomical sites (3). In the present study, we analyzed clinical strains that were recovered during a methicillin-susceptible S aureus (MSSA) outbreak in neonatal intensive care. Can J Infect Dis Med Microbiol 2012;23(3):130-134.

Key Words: Caenorhabditis elegans; Double-blinded test; Methicillin-resistant Staphylococcus aureus outbreak; MSSA; Neonatal intensive care unit; NICU; Staphylococcus aureus; Virulence host model

Staphylococcus aureus is an important human pathogen and has been a leading cause of nosocomial infection for many decades. It has been shown that S aureus is commonly identified in neonatal intensive care units (NICUs), with up to 80% of neonates colonized with the organism by day 10 in the NICU (1). Neonates in the NICU are more susceptible to S aureus infection due to their immature immune system and high frequency of use of invasive medical devices, particularly intravascular catheters. Therefore, S aureus, including methicillin-resistant strains (methicillin-resistant S aureus [MRSA]) have been frequently identified as causes of outbreaks of infection in NICUs.

HISTORIQUE : Le Caenorhabditis elegans a déjà été utilisé comme modèle hôte pour déterminer la virulence des isolats de Staphylococcus aureus résistant à la méthicilline. Dans la présente étude, les chercheurs ont étudié les souches de S aureus susceptible à la méthicilline (SASM) associées à une flambée dans une unité de soins intensifs néonatals (USIN), au moyen du modèle de C elegans.

MÉTHODOLOGIE : Les chercheurs ont repéré deux clones de flambée distincts au moyen de l’électrophorèse sur gel en champ pulsé, soit le SASM de type C et celui de type G, lors d’une flambée de SASM sur une période de sept mois à l’USIN du Sunnybrook Health Sciences Centre de Toronto, en Ontario. Le SASM de type C s’associait à une grave infection, tandis que celui de type G s’associait à une maladie moins invasive. Les chercheurs ont envoyé à Calgary, en Alberta, quatre isolats de type C représentatifs, trois de type G et trois isolats colonisés chez des nourrissons non liés à cette flambée, pour faire effectuer des tests de virulence à double insu dans le modèle hôte de C elegans et obtenir une meilleure caractérisation moléculaire.

RÉSULTATS : Les souches de flambée invasive (type C) ont démontré une activité nématicide élevée, les souches de flambée non invasive (type G), une virulence intermédiaire et les isolats de colonisation non liés à la flambée, une avirulence ou une virulence faible dans le modèle de C elegans, ce qui se traduit par des taux de suppression moyens de 93,0 %, de 61,0 % et de 14,4 % le jour 9, respectivement, dans ces trois groupes de souches. Divers groupes de souches de SASM possédaient leur propre profil génétique unique et des profils géniques de virulence, mais tous les isolats du même groupe (type C ou type G) partageaient des caractéristiques génétiques identiques et des schémas de virulence génétique.

CONCLUSIONS : La présente évaluation en insu a démontré que les activités nématicides des souches de SASM étaient bien corréllées avec la manifestation clinique lors d’une flambée de SASM à l’USIN, ce qui étaye la solidité du C elegans comme modèle hôte pour étudier la pathogénicité du S aureus.
outbreak that occurred in an NICU, using the C elegans model. We observed a high correlation between nematocidal activity (indicating enhanced virulence) and the presence of a clinical syndrome compatible with invasive infection.

METHODS
An outbreak and identification of isolates
Sunnybrook Health Sciences Centre is an 1100-bed, university-affiliated, tertiary-care, teaching hospital in Toronto, Ontario. It is a regional high-risk maternity referral centre with 29 level 3 NICU beds and 12 level 2 beds. MSSA strains were isolated from neonates in the NICU during the outbreak (December 2006 to June 2007) and during the precoule period (January to November 2006). Invasive infection was diagnosed in infants with a compatible clinical syndrome and in whom S aureus was recovered from blood cultures or from some other normally sterile site. Noninvasive infection was believed to be present in neonates with signs and symptoms of infection, who had isolation of S aureus from a nonsterile body site (eg, endotracheal aspirate, eye, skin/soft tissue abscess). S aureus colonization was determined to be present if the organism was isolated from a nonsterile site in a neonate without signs or symptoms of infection, or if it was obtained during culture surveillance as part of the outbreak investigation. Strain M92 is a nematode avirulent control strain and USA300-2406 is a virulent positive-control strain, both of which originate from Calgary, Alberta (3).

C elegans virulence assay
A representative sample of isolates was investigated for strain virulence using the C elegans host virulence model, performed as previously described (3). Briefly, Bristol N2 C elegans nematodes were maintained at room temperature (RT) on nematode growth medium (NGM) plates. For survival assays, 30 nematodes were transferred from NGM plates to a tryptic soy agar (TSA) plate grown with one testing MSSA strain and monitored for survival every 24 h. Plates with heat-killed bacteria were prepared as previously described (3). The experiments were repeated at least three times. Using GraphPad Prism (GraphPad Software, USA), nematode survival rate data were analyzed by the Kaplan-Meier method, and comparison of significant survival difference used the log-rank test. For worm proliferation experiments, all worms were kept on their original plates without nematode transfer, and the accumulated numbers of live larva and adult nematodes were estimated by counting the live worms outside of the bacterial lawn. The C elegans model was assayed by trained technologists ‘blinded’ to the clinical or epidemiological data, and to the pulsed-field gel electrophoresis (PFGE) typing results.

Quantification of bacterial in vitro growth and in vivo burden
In vitro bacterial growth curves were constructed by reading optical density at 600 nm (OD600). Briefly, overnight brain heart infusion (BHI) bacterial cultures were diluted 1:1000 in fresh BHI broth, and 200 µL of the culture was loaded onto a 96-well plate. Each well was covered with mineral oil. Plates were incubated at 37°C and the OD600 values were measured every 20 min using a Wallace Victor2 multilabel counter (Perkin Elmer, USA). For quantification of in vivo bacterial burden of C elegans, the nematodes were fed representative MSSA isolates, and bacteria were quantified by serial dilution and plating on days 1, 2, and 4. Briefly, eight worms from each tested strain at each time point were washed with phosphate buffered saline (PBS) six times, by sequentially soaking and transferring worms in six 50 µL PBS pools on a TSA plate. The washed worms were then transferred into 100 µL PBS in a 1.5 mL microtube and homogenized. Serial dilutions of the homogenized solutions were then plated to count the live bacteria.

Phenotypic and genotypic characterization of isolates
All S aureus isolates were typed by PFGE according to the Canadian standardized protocol (4). The strains were tested for the presence of Panton-Valentine leukocidin (PVL) genes, and 34 other common S aureus virulence genes, by polymerase chain reaction assay (3). The isolates were characterized by multilocus sequence typing (MLST), staphylococcal protein A (spa) typing and accessory gene regulator (agr) typing as previously described (5).

RESULTS
The outbreak of MSSA infections in an NICU
During a seven-month period (December 2006 to June 2007), 21 infants in the NICU were found to be infected with MSSA. Prevalence (colonization) screens of the nose, umbilicus and groin identified 28 additional infants found to be colonized with MSSA. Following a chart review, 10 additional MSSA infections were found to have occurred during an 11-month period (January to November 2006) before this outbreak (Figure 1A).

PFGE was performed on all but two outbreak isolates (n=47), and all 10 precutout isolates. Two distinct outbreak clones were identified by PFGE. Twelve patients were shown to be infected with a strain designated MSSA-C. This strain was associated with more severe infection and disease, accounting for three of four bloodstream infections that occurred during the outbreak period (Figure 1B). Another strain designated MSSA-G, which appeared to be associated with less invasive disease, was identified in six infants, two of whom were merely colonized (Figure 1B).
As expected, several other strains of MSSA were also recovered from neonates (n=21), most of which represented asymptomatic colonization (n=18) (Figure 1B). The majority of these strains had distinct PFGE profiles and were determined to be unrelated to the outbreak.

Nasal swab cultures were performed on NICU staff members early on in the outbreak investigation. Seven employees were infected with the MSSA-C strain but all were asymptomatic. No employee was positive for the MSSA-G strain. With the knowledge that transmission was in fact occurring between health care workers and patients, additional infection control interventions were implemented. As a result, no further transmission occurred within the unit and the outbreak was declared over in June 2007.

Four representative isolates of MSSA-C, three of MSSA-G and three infant-colonized isolates unrelated to the outbreak as determined by PFGE were found, coded as MSSA1 to MSSA10. MSSA1, MSSA2 and MSSA3 were the isolates responsible for three bacteremias in newborns, and MSSA4 caused a skin infection; all of the infections required treatment. MSSA5 was isolated from an umbilical site and MSSA6 and MSSA7 were both isolated from umbilical and eye sites. MSSA8 to MSSA10 were nonoutbreak isolates that were found to colonize on the endotracheal tube, nose or umbilical site. All isolates were submitted for the virulence assays in the C elegans host model and for further molecular characterization.

Double-blinded virulence tests in the C elegans host model
The virulence of the MSSA isolates was determined using the C elegans host model. Isolates MSSA1 to MSSA4 demonstrated nematocidal activity after 24 h. For MSSA1 to MSSA4, killing was noted, respectively, in 45.8%, 72.5%, 73.3% and 68.1% of worms by day 3 and 70.2%, 97.5%, 100% and 94.9% by day 6 (Figure 2A). There were no significant differences between MSSA2 to MSSA4 isolates over the nine-day experiment period (all P ≥ 0.13) with nematocidal activity similar to the virulent positive control strain USA300-2406, while MSSA1 showed relatively less virulence. In contrast, strains MSSA5 to MSSA7 showed similarly intermediate nematocidal activity with mean killing rates of 26.7%, 23.4% and 26.1% by day 3, and 56.5%, 59.1% and 59.4% by day 6, respectively. There were still 37.0%, 39.4% and 40.6% of worms surviving on day 9, respectively (Figure 2A). However, MSSA8 to MSSA10 showed avirulence or low nematocidal activity. MSSA8 was similar to the negative control colonization strain M92 and did not kill nematodes. Isolates MSSA9 and MSSA10 killed 15.0% and 4.0% by day 3 and 33.8% and 9.4% by day 9.
respectively. In addition, differential effects of different MSSA strains on the larval stage worms were observed. Both strains MSSA-C (MSSA1 to MSSA4) and MSSA-G (MSSA5 to MSSA7) efficiently inhibited larval proliferation, with less than 100 larvae observed on the tested plates on day 9. However, strains MSSA8 to MSSA10 produced a large number of second- and third-generation larvae, accumulating more than 1000 worms on day 9 (data not shown). The experiments suggested that MSSA1 to MSSA4 were highly virulent, MSSA5 to MSSA7 were immediately virulent and that MSSA8 to MSSA10 were avirulent or of low virulence in the C elegans model. Interestingly, only when these results were subsequently compared with clinical data was it determined that MSSA1 to 4 were the outbreak isolates associated with invasive infection (MSSA-C), MSSA5 to MSSA7 were associated with the noninvasive outbreak isolates (MSSA-G) and MSSA8 to MSSA10 were associated with the outbreak-unrelated colonization strains.

The nematocidal activities of the highly and intermediate virulent strains (MSSA1 to MSSA4 and MSSA5 to MSSA7) were abolished when the worms were fed with heat-killed bacteria (data not shown), suggesting that killing requires the presence of live bacteria. To determine whether the low or non-nematocidal activities of the MSSA strains were due to deficient bacterial growth, bacterial in vitro growth curves were constructed and the in vivo burden of representative strains were calculated. As shown in Figure 2B, all the strains except MSSA8, regardless of high or low virulence, showed similar growth curves. The avirulent strain MSSA8 exhibited a slower growth pattern than the other strains. Further comparisons of the bacterial in vivo burden on days 1, 2 and 4 did not denote any significant difference among the representative strains (MSSA8, MSSA5 and MSSA4) from the avirulence, intermediate and high virulence groups (P>0.34 [Mann Whitney test]) (Figure 2C).

**Molecular (genotypic) and phenotypic characterization of MSSA isolates.**

All 10 isolates were further characterized by MLST, spa, and agr typing in addition to PFGE (Figure 3). All MSSA-C isolates (MSSA1 to MSSA4) were clustered together by PFGE and carried the same MLST type (ST109), spa type (t209) and agr type (II), whereas all MSSA-G strains (MSSA5 to MSSA7) shared identical PFGE patterns and genetic profiles (MLST type ST5, spa type t071 and agr type II). The other three colonization strains each had a unique genetic profile. Strain MSSA8 was MLST type ST942, spa type t445 and agr type III. Strain MSSA9 was MLST type ST45, spa type t1156 and agr type I, and strain MSSA10 was MLST type ST8, spa type t334 and agr type I (Figure 3).

PFGE and strain MSSA8 harbored nearly the same genetic traits, suggesting that the potential to kill the nematode gut was not influenced by the genetic background. In addition, other virulence genes (MSSA8, MSSA5 and MSSA4) from the avirulence, intermediate and high virulence groups (P>0.34 [Mann Whitney test]) (Figure 2C).

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**DISCUSSION**

Invertebrates have been used as host models for studying the virulence of pathogens because of their convenience and simplicity. However, such models may not accurately reflect the interactions between humans and microorganisms because invertebrates are distantly related evolutionarily. It is known that some genes involved in the innate immune responses of invertebrates and mammals are highly conserved (6). Sifri et al. (7) showed that the host p38 MAP kinase pathway, which is involved in human neutrophil antimicrobial response, is also important for C elegans resistance against S aureus infection. Similarly, certain key virulence factors of S aureus, such as agr, hla and spa, are commonly required for pathogenicity in both nematodes and mammalian hosts (7). Moreover, in previous reports on C elegans infection, the nematocidal activity of the clinical MRSA strains correlated well with clinical and epidemiological data collected in the Calgary Health Region over a six-year period (3). In the current blinded investigation, we observed a high degree of correlation between nematocidal activity (by MSSA strain virulence) and the clinical scenario corresponding to the outbreak setting with respect to invasive and noninvasive infections versus colonization. Together, these results strongly support the use of C elegans as a robust host model to study the virulence of S aureus strains.

The nematocidal activities of high-virulence MSSA strains required the presence of live bacteria, as heat-killed bacteria lost their virulence, which was consistent with previous studies (3,7). However, all MSSA strains, regardless of high or low nematocidal activities, had similar in vitro growth curves, suggesting that the bacterial in vitro growth pattern does not exert an influence on bacterial nematocidal activities. Although the avirulence strain MSSA8 had a lower in vitro growth rate, it showed a similar colonization and replication ability within the nematode gut as the high and intermediate virulence strains MSSA4 and 5. These results suggest that bacterial virulence factors from live bacteria, other than bacterial replication, are involved in nematocidal activities.

Our previous findings suggested that the virulence of MRSA in C elegans may be determined by the specific genetic background of the MRSA strain (3). In the present study, the high (MSSA1 to MSSA4), intermediate (MSSA5 to MSSA7) and low (MSSA8 to MSSA10) nematocidal MSSA strains possessed unique genetic characteristics.
and distinct virulence gene profiles, which further supports the above conclusions. MSSA1 belonged to the invasive outbreak strain (MSSA-C) and shared identical genetic and virulence gene profiles with the other isolates (MSSA2 to MSSA4) within the strain group (Figure 3). However, MSSA1 demonstrated slightly less nematocidal activity than its counterparts MSSA2 to MSSA4 (the mean killing rate was 70.2% versus 97.5% over a nine-day experimental course, respectively) (Figure 2A), indicating that it may have some degree of unrelatedness. Interestingly, the antibiotic resistance tests revealed that MSSA2 to MSSA4 isolates were resistant to clindamycin and erythromycin, while the MSSA1 strain was susceptible to both of these agents (data not shown). This further supports the hypothesis that the MSSA2 to MSSA4 isolates may have acquired genes mediating resistance to these agents in addition to other virulence gene(s) favoring their propensity for dissemination, invasiveness and evolution. Comparison of the virulence gene profiles of the virulent (MSSA-C) and intermittently virulent (MSSA-G) strains demonstrated that some virulence genes, such as the exotoxin gene chp, adhesive molecular gene sdrC, and exoenzyme genes hysA and sak, were missing from the MSSA-G strains but not the MSSA-C strains, implying that these genes may impart the ability to cause invasive infection. However, some of these genes were present in the avirulent (or low virulent) MSSA8 to MSSA10 strains. In addition, the toxic shock syndrome (tst) gene has been shown to be associated with an increase in the incidence of bloodstream infections (8). PVL genes have also been suggested to be one of the major virulence factors associated with severe infections due to S. aureus and the propensity of community-associated MRSA strains to disseminate (9,10). Only one MSSA8 isolate in the current study carried PVL and tst genes, although that isolate was associated with colonization instead of infection and avirulence in the C elegans model. These results are in agreement with our previous finding that multiple virulence factors or certain combinations of virulence factors acting in concert may be responsible for the nematocidal activity (3). Further studies are needed to elucidate the elaborate mechanisms involved in C elegans killing (virulence) by S. aureus.

The present study was the first correlational observation validating the C. elegans host virulence model in characterizing the virulence of outbreak and nonoutbreak strains of S. aureus. The present study may be limited by its relatively small sample size, as well as the possibility of misclassification of invasive versus noninvasive isolates based on clinical criteria. In summary, we have demonstrated that the C. elegans host model provides a validated approach to studying S. aureus virulence determinants and could lead to a better understanding of bacterial pathogenesis in staphylococcal infections.

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