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An investigation and evaluation on species and characteristics of pathogenic microorganisms in Chinese local hospital settings

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ABSTRACT

There are currently great concerns about the level of bacterial contamination in hospitals, as well as resistance to antimicrobial agents. The species and characteristics of microbes in Chinese hospitals are closely related to healthcare safety and the prevention and control of infections. However, data on the exposure of patients to microbes in Chinese hospitals are limited. The present study investigated the genera of microorganisms in Chinese hospitals. We evaluated their characteristics, such as antibiotic susceptibility, tolerance to disinfectants, and toxicity, using silkworms (*Bombyx mori*) as an animal model. Twenty-six distinct bacterial strains were isolated, and their genera were determined by sequencing their 16S rDNA regions. Twenty-five strains were resistant to one or more antibiotics, and six strains were resistant to multiple antibiotics. The results of minimal inhibitory concentration testing showed that eight strains were resistant to a chlorine-containing disinfectant, and 12 strains were resistant to Povidone–iodine. Following the injection of bacterial cultures into the silkworm hemolymph, 15 strains killed all of the silkworms within 5 d. Additionally, bacterial strain 14 killed all of silkworms within 12 h with a median lethal dose of 4×10^4 colony-forming units/larva. This study provides useful information for healthcare safety in Chinese hospitals.

1. Introduction

Bacterial contamination and antimicrobial resistance in hospitals have raised increasing concerns in the medical and healthcare areas [1–3]. A study of device-associated, healthcare-associated infection rates and microorganism profiles in a hospital in Shanghai, China, showed that the overall infection rate was 5.3%, and the number of infections per 1000 intensive care unit (ICU)-days reached 6.4%. The most commonly isolated microorganism was *Acinetobacter baumannii*, followed by *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, and *Staphylococcus aureus* [4]. Compared with other member countries of the International Nosocomial Infection Control Consortium, the situation in China is worse than that in developed countries [5]. Conditions in hospitals and communities in China, the largest developing country in the world, have greatly improved over the past few years, and research on pathogens in healthcare settings has achieved increasing prominence. However, information about pathogenic microorganisms in Chinese hospitals is not widely available in English language journals.

Disinfection is the most fundamental and crucial countermeasure to clean hospitals and to prevent and control hospital infections [1]. However, in the 1950s, Chaplin reported that *Serratia marcescens* survived in alkyldimethylbenzyl ammonium chloride [6]. Since then, many studies of microbial resistance to disinfectants have been reported, such as the resistance of *Listeria monocytogenes* to benzalkonium chloride [7], the resistance of *P. aeruginosa* to chlorophenol [8], and the resistance of *Clostridium difficile* to peroxygen, aldehyde, and chlorine releasing agents [9]. As organisms are present in a variety of clinical settings, the extensive use of broad-spectrum antibiotics and a lack of good stewardship have contributed to the increasing emergence of serious multidrug-resistant (MDR) infections. These infections predominantly include MDR *P. aeruginosa*, extended-spectrum beta-lactamase-

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producing Enterobacteriaceae, and MDR *A. baumannii* [10]. The levels of antimicrobial resistance vary between different types of healthcare facilities and different geographic areas.

The identification of pathogenic microorganisms, as well as toxicity tests, are generally performed using mammals. However, the use of a large number of mammals for infection experiments is associated with ethical problems and is costly as well [11]. The silkworm (*Bombyx mori*) is an easily bred invertebrate animal that is used for basic research studies. Silkworm larvae are large enough to inject sample solutions into their hemolymph, which is difficult in *Caenorhabditis elegans* and *Drosophila* larvae. Kaito et al. have reported a silkworm infection model in which silkworms were killed by injecting them with microorganisms that are virulent in humans, such as *S. aureus*, *P. aeruginosa*, *Vibrio cholerae*, and pathogenic strains of *Escherichia coli*, but they were not killed by non-pathogenic strains of *E. coli*. Moreover, the killing of silkworm larvae by *S. aureus* could be counteracted by clinically effective antibiotics [12]. The silkworm model has been used to identify *S. aureus* virulence genes [13]. It has also been reported that silkworms are useful for evaluating the pathogenicity of a *Bacillus* soil bacterium, as well as for purifying an exotoxin secreted from this bacterium by monitoring its toxicity [14]. Another study used silkworms to test the virulence of 122 strains of bacteria isolated from the intestines of fish and shellfish at 37 °C. Its findings suggested that bacterial pathogenicity against mammals can be predicted based on measurements of their silkworm-killing activity [15]. Therefore, the silkworm model can be an effective alternative to a mammalian infection model for certain purposes.

The present study investigated the genera of microorganisms in a Chinese hospital, and evaluated their characteristics, such as antibiotic susceptibility and tolerance to disinfectants, and it assessed their pathogenicity using silkworms. The results will increase our understanding of the genera of pathogenic microorganisms, as well as their antimicrobial resistance, in local healthcare settings, and it will provide suggestions for the rational use of disinfectants and antibiotics, the effective removal of harmful pathogens, and better treatment options for bacterial infections.

2. Materials and methods

2.1. Isolation of hospital bacteria and species determination

One hundred and twenty surface samples from different units, such as the delivery room, ophthalmology room, operating theater, neonatal room, ICU, and gastroscopy room of the Fourth People's Hospital of Zhenjiang, Jiangsu, China, were collected randomly. Samples were suspended in sterilized water, and the supernatants were spread onto nutrient agar plates. Colonies were isolated after overnight incubation.

The 16S rDNA region was amplified by colony polymerase chain reaction (PCR) (95 °C for 5 min, followed by 30 cycles of 95 °C for 45 s, 55 °C for 45 s, and 72 °C for 100 s, followed by 10 min at 72 °C) using primer pairs 27F (5′–AGAGTTTGATCMTGGCTCAG–3′) and 1492R (5′–TACGGYTACCTTGTTACGACTT–3′) [16], and sequenced subsequently. A Basic Local Alignment Search Tool (BLAST) search against the sequenced 1500 ± 250 bp of the 16S rDNA region was performed to identify the bacterial genera. The 16S rDNA sequences—which were more than 99% identical to another bacterial sequence—were used for the genus determination.

2.2. Antibiotic sensitivity test

Antibiotics were selected to represent the main classes of antibiotics that are widely used for human therapy. Ampicillin (Amp), kanamycin (Kan), gentamicin (Gen), tetracycline (Tet), and chloramphenicol (Chl) were obtained from Generay Biotech Co., Ltd. (Shanghai, China), and they were prepared in accordance with the manufacturer's instructions. Antibiotic susceptibility was determined by the standard broth dilution method [17]. Briefly, serial two-fold dilutions of each antibiotic were incubated with bacterial suspensions that were adjusted to 5×10^5 colony-forming units (CFU)/ml in Mueller–Hinton Broth (Sigma–Aldrich, St. Louis, MO, USA). Growth and sterility controls were incubated at 37 °C for each isolate. The results were interpreted according to recommendations from the Clinical and Laboratory Standards Institute (CLSI) after 24–48 h of incubation.

2.3. Minimum inhibitory concentration and minimum bactericidal concentration of disinfectants

Two kinds of disinfectants that are used daily in healthcare settings were selected: 84 disinfectant solution containing chlorine (50 g/L) (Anjie, De Zhou, China) and Povidone–iodine (PVP–I) containing iodine (5 g/L) (Anjie). *E. coli* American Type Culture Collection (ATCC) 25922, *S. aureus* ATCC 6538, *P.* and *Bacillus subtilis* ATCC 9372 (European Standard BSEN 1276: 1997, suspension test strains) were used in this study. Preliminary tests were conducted to determine the most effective neutralizer for each product. The neutralizers used in the bactericidal tests were as follows: 1% sodium thiosulfate (w/v) for the 84 disinfectant solution, and 0.5% sodium thiosulfate (w/v), 1% lecithin (w/v), and 1% Tween-80 (v/v) for PVP–I.

The minimal inhibitory concentration (MIC) and minimal bactericidal concentration (MBC) were determined by the double broth dilution method. To determine the MICs, 5 ml of a serially diluted disinfectant solution was mixed with 5 ml of double nutrient broth in sterile tubes. Then, 0.1 ml of a bacterial suspension was added to adjust the bacterial concentration to 5×10^5–5×10^6 CFU/ml. Control tubes of nutrient broth containing only bacteria, as well as nutrient broth containing only a single disinfectant, were also prepared. All tubes were incubated at 37 °C for 24–48 h. All experiments were repeated at least twice, and triplicate determinations were made. Any bacterial growth was compared to the growth of the controls and recorded, and the concentration of the lowest dilution that inhibited bacterial growth was interpreted as the MIC.

Assays of MBCs were conducted on the basis of the MIC results. Two ml of sterile nutrient broth containing neutralizers was added to the tubes that showed no bacterial growth, and results were examined after incubation at 37 °C for 24 h. All experiments were repeated at least twice, and triplicate determinations were made. Any growth in the tubes was recorded, in which the concentration of the lowest dilution was interpreted as MBC.

2.4. Evaluation of the toxicity of hospital bacteria using silkworms

The silkworm infection experiments were performed in accordance with previously established methods [13]. Silkworm strain 306 was raised from fertilized eggs to fifth-instar larvae in our laboratory. The newly exuviated fifth-instar larvae were fed with antibiotic-free mulberry leaves for 1 d. Then, a total of 5 μl of an overnight culture of the hospital bacteria strains was injected into the silkworm hemolymph through the intersegment membrane using a sterile microsyringe (Shanghai Microplate Co. Ltd., Shanghai, China). Pressure was immediately applied for 10 s at the injection site using alcohol wipes to stop any bleeding. Control larvae were injected with saline (0.6% NaCl). The larvae were maintained at room temperature and used for further analysis. Each experimental group contained 10 silkworm larvae, and the number of living silkworm larvae was counted every 12 h. All