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Characterization of predominant bacteria isolates from clean rooms in a pharmaceutical production unit*

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Abstract: Aims: To screen for the predominant bacteria strains distributed in clean rooms and to analyze their phylogenetic relationships. Methods and Results: The bacteria distributed in air, surfaces and personnel in clean rooms were routinely monitored using agar plates. Five isolates frequently isolated from the clean rooms of an aseptic pharmaceutical production workshop were selected based on their colony and cell morphology characteristics. Their physiological and biochemical properties, as well as partial 16S rDNA sequences, were analyzed. Results showed that all the five isolates belong to Gram positive bacteria, of which three were *Staphylococcus*, one *Microbacterium* and one *Bacillus* species. Sensitivity tests for these bacteria isolates to 3 disinfectants showed that isolate F03 was obtuse, and had low susceptivity to UV irradiation, while isolates F02, F01 and F04 were not sensitive to phenol treatment. Isolates F04, F01 and F05 were resistant to chlorhexidine gluconate. Conclusion: Bacteria widely distributed in clean rooms are mainly a group of Gram positive strains, showing high resistance to selected disinfectants. Significance and impact of the study: Clean rooms are essential in aseptic pharmaceutical and food production. Screening bacteria isolates and identifying them is part of good manufacturing practices, and will aid in finding a more effective disinfection method.

Key words: Clean room, Bacteria, 16S rDNA, Phylogenetic tree, Disinfectant, Resistant abilities
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INTRODUCTION

Clean rooms are essential in aseptic pharmaceutical or food production. Monitoring microbial distribution and identifying the predominant isolates is part of good manufacturing practices (Akers, 1997). The commonly used protocol for monitoring involves the use of media such as soybean casein digest agar (SCDA) and incubation at 30 °C for 4 d (Akers, 1994). According to the European Union's good manufacturing practice directive, the permissible number of colony forming unit (CFU) on surface contact plates for grades A and B is 1 and 5 respectively (Schicht, 1998). The number permitted by USP (US Pharmacopeia) for classes 100 and 10000 is 3 and 5 respectively (USGSA, 1992). To meet such requirements, all the surfaces within the clean room, air, floor and personnel hands, are disinfected routinely using a variety of disinfectants. Ultraviolet (UV) irradiation, Lysol (phenol) solution swabbing and Maskin (chlorhexidine gluconate, CHG) solution immersion are three widely used ways for disinfecting clean room air, furniture surface and personnel skins in many pharmaceutical factories.

Maintaining the integrity of a clean room is a constant battle. To decide which method, or combination of methods, to be employed in disinfecting aseptic workshop, there is a need to understand the kind of bacteria that are the prime sources of contamination (Nagarkar *et al*., 2001). Therefore, knowledge of the microbial diversity of clean rooms, as well as any extreme characteristics these microbes might possess, is essential to the development of disinfection technologies. The aim of this study was to isolate and identify the predominant bacteria strains distributed in clean rooms of a pharmaceutical

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workshop. In on-going investigations to determine and document possible microbial contamination in clean rooms, bacteria strains were isolated and their colony and cell morphology characteristics were compared. To analyze the phylogenetic relationships of the predominant isolates, the 16S rDNA genes were amplified and their partial sequences were determined. To determine the extreme characteristics of these isolates, their resistance levels to 3 disinfectants (UV, phenol and CHG) were tested.

MATERIALS AND METHODS

Bacteria isolation

Bacteria strains were isolated in the routine aseptic monitoring courses from the clean rooms of a pharmaceutical factory, using settle plate counts for air flora, swabs from a variety of surfaces in the working area, and finger dabs of the working personnel after routine disinfection procedures (MHPRC, 2002). For air sampling, SCDA (casein hydrolysate, 1.5 g; soybean digest, 0.5 g; sodium chloride, 0.5 g; agar, 1.5 g; and distilled water to make 100 ml, pH 7.3) plates were exposed in the clean room after UV irradiation. For surface sampling, a 25-cm^2 area of floor was swabbed by tampon after disinfection with 5% (w/v) phenol solution. The hands of workers were sampled after disinfection with 1% (w/v) CHG solution. Hands were washed using the axenic water to collect the bacteria. All the plates were cultivated at 30 °C for 96 h, and bacteria were purified further using streaking method.

Morphological and biochemical characterization of predominant bacteria

Representative bacteria strains were selected based on colony morphology. On divergent colonies, Gram staining, as well as oxydase and catalase tests was performed. The method of these tests followed John *et al*.(1994). With this process, and based on the distribution frequencies of these isolates, 5 predominant strains named F01~F05 were selected. Their cell morphologies were further observed under JEM-1200EX transmission electron microscopy (JEOL, Japan) with uranyl acetate staining, and physiological/biochemical characteristics were tested according to John *et al*.(1994).

Molecular characterization of the predominant bacteria

DNA was extracted from 5 isolates with freezing and thawing method (Wu and Zhou, 2005). Polymerase chain reaction (PCR) amplification was conducted in Eppendorf Mastercycler using 341F and 907R as primers (Teske *et al*., 1996). The PCR amplification mixture contained 0.2 mmol/L (each) dNTP, 400 nmol/L (each) primer, 5 mmol/L $MgCl_2$, and 1 U Taqplus (Bioasia, China) in a final volume of 50 μl. After a hot start at 94 °C for 3 min, 30 cycles of PCR reaction were run as follows: denaturation at 94 °C for 1 min, annealing at 55 °C for 45 s, and extension at 72 °C for 1 min. In addition, a final extension at 72 °C for 10 min was added. The resulting products were analyzed by electrophoresis in 1.0% agarose gel and purified with QIAquick PCR purification kit column (Qiagen, Germany). Sequences were determined in an ABI PRISM 3730 DNA automated sequencer using 341F as a sequencing primer, and their closest matches were found by blasting against the short and nearly exact matches from NCBI (National Center for Biotechnology Information) databases (http://www.ncbi.nlm.nih.gov). Sequences were aligned and the phylogenetic tree was generated using DNAMAN package (Lynnon Biosoft, Canada) with evolutionary distances method (bootstrapping 1000 trials). The determined partial 16S rDNA sequences described in this study were deposited in European Molecular Biology Laboratory (EMBL) nucleotide sequence database under accession Nos. AM268420 to AM268424.

Preparation of bacteria cell suspension

The isolated bacteria were inoculated into nutrient liquid (beef extract 0.5%, peptone 1%, NaCl 0.5%, pH 7.2) and cultivated in a rotating shaker (200 r/min) at 30 °C for 12 h, 5 ml fermentation liquid was centrifuged at 10000×g for 5 min, then the precipitated cells were washed twice and diluted to 10^6 cells/ml with sterilized distilled water.

Effect of UV irradiation on survival rate of bacteria isolates

A 15 W UV light [irradiation intensity is 30 μJ/(cm^2·s)] was used to determine the UV resistance abilities of the isolated strains. After turning on the UV light for 30 min to stabilize the irradiation inten-

sity, 5 ml cell suspension was placed into a sterilized disk, exposed to UV light (20 cm) for 0~5 min per half minute, with slow stirring using a magnetic stirring apparatus, using a serial dilution method and then the surviving cells were counted on nutrient agar plates (Park and Noguera, 2007). All procedures were performed under red light.

Effect of phenol and CHG solution on survival rate of bacteria isolates

Two methods were used to determine the disinfection effects of phenol or CHG solution on the isolated bacteria. The first one, named short term treatment test, was to inoculate 10^7 bacteria cells into 5 ml at various concentration of the disinfectants and was kept at 30 °C for 30 min. The cells were then collected by centrifugation and washed twice with sterilized distilled water to remove adherent phenol or CHG. Surviving bacteria cells were counted on nutrient agar using colony forming unit method. The second method, named long term co-culture test, was to cultivate bacteria cells in liquid nutrient with varying contents of disinfectants for 48 h. Bacteria growth was determined using colorimetry based on an increase in absorbance at 650 nm wave length.

Statistical analysis

All the disinfectant resistance tests were performed in triplicate. Since there was no interaction between the isolates and disinfectants, two-ways analysis of variance (ANOVA) were performed to determine the treatment differences between the isolates and *Staphylococcus aureus* (Sa) using statistical software in Microsoft Excel.

RESULTS

Bacteria isolation and characterization

Bacteria were isolated in the routine microbiological monitoring processes for clean rooms of a pharmaceutical factory. Based on the colony and cell morphology, as well as distribution frequency and sampling origin, 5 predominant bacteria strains named F01~F05 were selected. Isolates F01, F02 and F04 are cocci, about 0.5 μm in diameter, while isolates F03 and F05 are rods. In addition, isolate F05 has an endospore and several flagella (Fig.1). Further investigations showed that all the 5 isolates are positive in Gram staining reaction, and have no mobility except isolate F05.

The biochemical and physiological tests also showed that isolates F01, F02 and F04 are similar in most of the properties (Table 1), and 16S rDNA sequence analyses further demonstrated that these 3

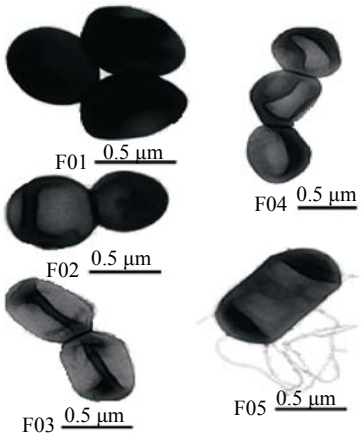

Fig.1 Transmission electron micrograph of 5 isolates. Bacteria cells were directly stained with uranyl acetate and observed under JEM-1200EX electron microscopy

Table 1 Biochemical and physiological characterization of the predominant bacteria isolates

Biochemical and physiological properties	F01	F02	F03	F04	F05
Catalase test	+	+	+	+	+
Oxidase test	−	−	−	−	−
Amylase test	−	−	−	−	−
V-P test	+	+	−	−	−
Indole production	+	+	−	+	−
Nitrate reduction	−	−	−	−	+
Citrate utilization	−	−	−	+	−
Gelatin liquefaction	+	+	+	+	−
Coagulation and acid production in litmus milk test	−, −	−, −	−, +	−, −	−, +
Acid and gas production by fermentation of					
Glucose	+, −	+, −	+, −	+, −	+, −
Lactose	+, −	+, −	+, −	+, −	+, −
Xylose	−, −	+, −	+, −	−, −	+, −
Sorbitol	+, −	−, −	−, −	+, −	−, −
Maltose	+, −	−, −	−, −	+, −	−, −
Mannitol	+, −	−, −	−, −	+, −	−, −
Sucrose	−, −	+, −	+, −	−, −	−, −
Growth on nutrient agar with					
7.5% NaCl	+	+	+	+	+
10% NaCl	+	+	−	+	+
15% NaCl	−	−	−	−	+

isolates share more than 99% similarities. Blasting analyses of partial 16S rDNA sequences confirmed that these 3 isolates belong to the genus *Staphylococcus*. However, molecular characterizations were at the limits of resolution for the differentiation of species in this genus. Combined with the results of biochemical and physiological tests (Table 1), it can be preliminarily concluded that F01 and F04 closely match *S. saprophyticus* and *S. cohnii* subsp urealyticus, respectively, and F02 may be *S. warneri* or *S. pasteuri*. With the same methods, F05 was identified as a Firmicutes *Bacillus fusiformis*, while F03 was an Actinobacteria *Microbacterium oleivorans* (Fig.2).

Effect of UV irradiation on the growth of the isolates

UV irradiation is a widely used method for disinfection of clean room air in pharmaceutical factories. Isolate F03 had much higher UV resistance than the other strains. When exposed to UV rays for 3 min, 7% of suspension cells were still alive, while only about 4% of the F05 cells survived after being irradiated for 1 min (Fig.3). However, it cannot be simply deduced that isolate F05 is more sensitive to UV rays than the other 4 isolates because the irradiated cells in this study were in the state of log phase, when endospores have not yet been formed. In 3 isolates belonging to *Staphylococcus*, F01 and F04 exhibited weaker UV resistance than the control *S. aureus* (Sa), while the contrary was true for the isolate F02. However, differences among these strains are not notable (Table 2), demonstrating that the 3 isolates are the general strains distributed in natural environments and not the UV-resistant mutants.

Table 2 *P*-value in *F*-test on the survival rates between isolated bacteria and *Staphylococcus aureus*

Compared strains	UV irradiation	CHG ST treatment	CHG LT co-cultivation	Phenol ST treatment	Phenol LT co-cultivation
F01-Sa	0.374	0.129	0.148	0.777	0.028
F02-Sa	0.309	0.057	0.858	0.466	0.130
F03-Sa	0.098	0.106	0.041	0.351	0.211
F04-Sa	0.374	0.287	0.363	0.842	0.025
F05-Sa	0.374	0.100	0.106	0.074	0.270

P-value: Probability value, $0.01<P<0.05$, significant difference; $P>0.05$, no significant difference. CHG: Chlorhexidine gluconate; ST: Short term; LT: Long term; Sa: *Staphylococcus aureus*

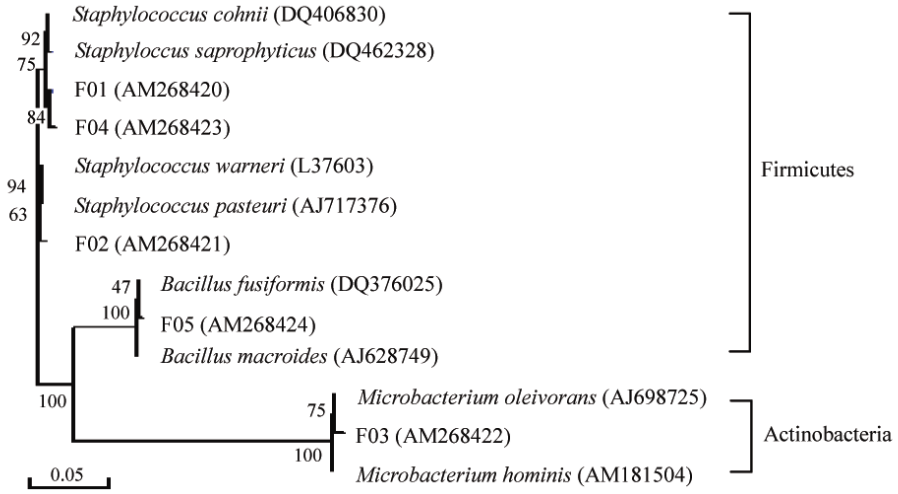

Fig.2 Phylogenetic affiliation of the bacteria isolated from clean rooms as revealed by comparative analysis of partial 16S rDNA sequences and those stored in public nucleotide databases. F01 to F05 are sequences determined in this study. Accession numbers are noted in brackets. The sequences were aligned and phylogenetic tree was constructed by DNAMAN package. Division level groupings are bracketed at the right of the figure. The scale-bar represents 5% estimated sequence divergence. Numbers indicate bootstrap confidence values as the percentage of 100 bootstrap replications

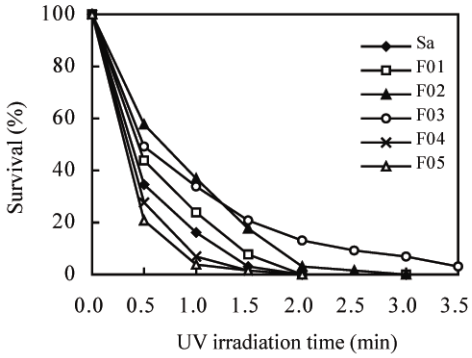

Fig.3 Effect of UV irradiation on the survival of the isolates
Sa: *S. aureus*

Effect of phenol solution on the survival of isolates

From the median lethal dose (LD_{50}) of the phenol solution on the 5 isolates (Fig.4), phenol resistance abilities were as follows: F02>F01>F03>Sa>F04>F05 in the short term treatment tests and F04>F02>F01>Sa>F05>F03 in the long term co-culture tests. Similar results were obtained when evaluated with 90% lethal dose (LD_{90}), that is: F02>F01>F04>F05>Sa>F03 in the short term treatment tests and F04>F01>F02>F05>Sa>F03 in the long term co-culture tests. This leads to the conclusion that isolates F02 or F01 are less sensitive, whereas isolate F03 is sensitive to phenol treatment. Isolate F05 is special in that more than 60% of cells were killed at 0.1% phenol treatment solution for 30 min. However, 3.1% and 0.25% of cells were still alive under treatments with 1% and 2% phenol at the same conditions. It seems that endospores had been formed in a small proportion of test cells.

It is odd that at low phenol concentration (for example, 0.1%), survival rates of the 3 isolates belonging to *Staphylococcus* (F01, F02 and F04) are higher in the long term co-culture tests than in the shot term treatment tests. Different culture methods in the two kinds of tests may be one of the reasons. It seems that some injured bacteria cells that could not recover on nutrient agar could still grow in liquid media, making the optical density of the culture to increase slowly. Also the efficacy of the phenol may be reduced by high organic load in the co-culture test.

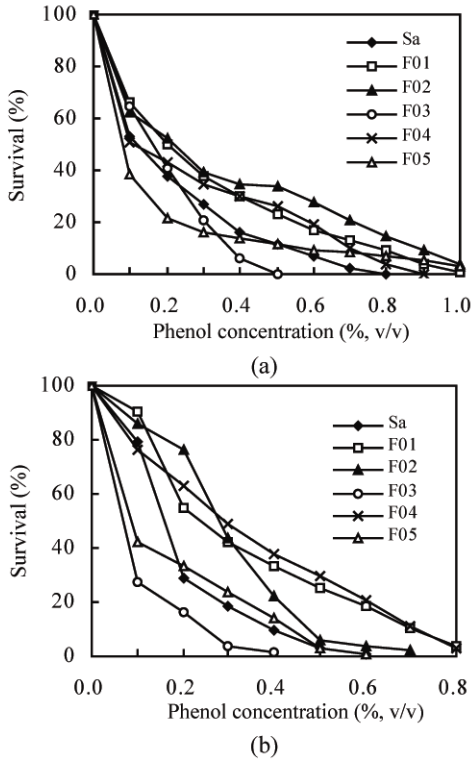

Fig.4 Effect of phenol solution on the survival of the isolates in a short term treatment test (a) and a long term co-culture test (b)
Sa: *S. aureus*

Effect of CHG solution on the survival of 5 isolates

The LD_{50} of the 5 isolates follows the order of F04>F05>F01>F03>Sa>F02 in short term treatment tests and F04>F01>Sa>F03=F05>F02 in long term co-culture tests. Similar results were obtained when evaluated with LD_{90}, that is: F04=F05>F01=F02>F03=Sa in short term treatment tests and F04=F01>Sa>F05>F02>F03 in long term co-culture tests (Fig.5). Isolates F04 and F01 had high resistance abilities while isolates F02 and F03 were sensitive to this disinfectant in both tests. Like on the effect of phenol solution, isolate F05 showed strong resistance to 30 min treatment with CHG solution, but was easily inhibited in co-culture tests. It seems that the endospore of F05 can endure 30 min treatment of CHG solution and therefore could recover on nutrient agar after the disinfectant was removed, whereas in long term co-culture test, low dosage of disinfectant was active during the entire culture period and the endospore was therefore not able to germinate.

Osmotic resistance tests (Table 1) show that 4 of the 5 isolates can grow on nutrient agar with 10% of NaCl and that the isolate F05 can even grow on 15% NaCl, suggesting that the 5 isolates have the abilities to resist severe environment pressures.

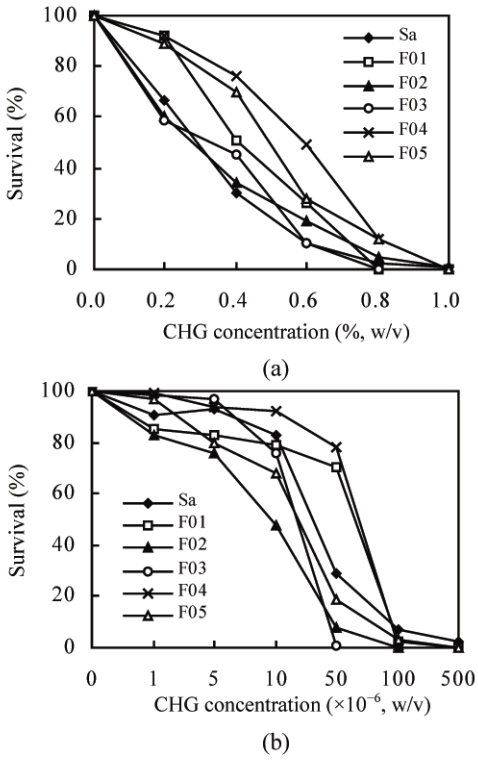

Fig.5 Effect of chlorhexidine gluconate (CHG) on the survival of the isolates in a short term treatment test (a) and a long term co-culture test (b)
Sa: *S. aureus*

Significant test of disinfectants resisting abilities between 5 isolates and *Staphylococcus aureus*

Although the 5 isolates have different resisting abilities to 3 disinfectants, two-ways ANOVA showed no significant differences between these 5 isolates and *S. aureus* (Table 2), suggesting that these isolates are just the normal bacteria in the environment, and that the resisting abilities are the inherent characteristics of these strains, rather than plasmid hold or DNA changed mutants.

DISCUSSION

Maintaining the integrity of a clean room is a constant battle (Nagarkar *et al*., 2001). There are 3 prime sources of contamination. The first is from human errors. To control this source of contamination, human hands must be washed with disinfectant. CHG, as a cationic surfactant, is the widely used skin disinfectant because of its mild nature. Contamination may also result from the room surface areas. To avoid such contamination, floors, walls and ceilings must be swept with phenol or other disinfectants. The third contamination source is from the room air. UV irradiation is the most convenient way to sterilize room air although it is not very penetrating and needs direct exposure.

To ensure a clean room conforming to the designated classification, constant monitoring of contaminant sources and identification of the predominant contaminant bacteria is usually necessary (Rosch *et al*., 2005). This study found that the predominant contaminant bacteria were a group of Gram positive bacteria: either spore-forming *Bacillus*, or non-sporulating *Staphylococcus* and *Microbacterium*. Further experiments showed that these bacteria could endure UV irradiation and phenol or CHG treatment. These results are in agreement with the findings of other workers (Onaolapo, 1995). Shaban *et al*.(1997) and Newcombe *et al*.(2005) found that *Staphylococcus* and spore-forming *Bacillus* were more resistant to UV than the other vegetative bacteria. Ajaz *et al*.(2004) screened 4 phenol resistant bacteria strains, two of which belong to genera *Staphylococcus* and *Bacillus*. Ogunniyi *et al*.(2000) also found that *Bacillus* and *Staphylococcus* were resistant to CHG treatment.

The mechanisms associated with resistance have received uneven attention. For most disinfectants, the studies are largely on phenomenological descriptions of the occurrence. Much less is known about the frequency with which resistance develops and the impact of environmental factors on resistance development (Chapman, 2003). This study found that all the 5 identified isolates were Gram positive bacteria, either spore-forming *Bacillus*, which is known to confer resistance to extreme environmental conditions, or non-sporulating *Staphylococcus* and *Microbacterium*, which have a thick cell wall. The thick wall of a cell or spore is a reasonable explanation for resistance to UV irradiation because this kind of non-ionizing radiation penetrates weakly. However, to phenol and CHG solutions, the cell wall could not be a reasonable explanation for retarding disinfectant entrance. It seems that physiological or genetic changes, such as phenotypic adaptation, genetic alteration, or genetic acquisition, must have been developed (Cloete, 2003). It has been reported that some genes special resistant phenotypes are located to-

gether on mobile genetic elements such as a plasmid, transposon, or integron (Chapman, 2003), and therefore, the development of resistance to one antibacterial agent is always accompanied by the appearance of resistance to another agent (Dukan and Touati, 1996). However, two-ways ANOVA showed no significant differences between the isolates and the control (Table 2), suggesting that these isolates are just phenotype adapted wild strains, rather than mutants resulting from gene acquisition.

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References

Ajaz, M., Noor, N., Rasool, S.A., Khan, S.A., 2004. Phenol resistant-bacteria from soil: identification-characterization and genetic studies. *Pakistan J. Botany*, **36**(2):415-424.

Akers, M.J., 1994. Parenteral Quality Control, 2nd Ed. Marcel Dekker Inc., New York.

Akers, J.E., 1997. Environmental monitoring and control: proposed standards, current practices, and future directions. *J. Pharm. Sci. Technol.*, **51**(1):36-47.

Chapman, J.S., 2003. Disinfectant resistance mechanisms, cross-resistance and co-resistance. *Int. Biodeter. Biodegr.*, **51**(4):271-276. [doi:10.1016/S0964-8305(03)00044-1]

Cloete, T.E., 2003. Resistance mechanisms of bacteria to antimicrobial compounds. *Int. Biodeter. Biodegr.*, **51**(4): 277-282. [doi:10.1016/S0964-8305(03)00042-8]

Dukan, S., Touati, D., 1996. Hypochlorous acid stress in *Escherichia coli*: resistance, DNA damage, and comparison with hydrogen peroxide stress. *J. Bacteriol.*, **178**(21): 6145-6150.

John, G.H., Noel, R.K., Peter, H.A.S., Bergy, D., 1994. Bergey's Manual of Determinative Bacteriology, 9th Ed. Williams & Wilkins, Lippincott.

MHPRC (Ministry of Health of People's Republic of China), 2002. Technical Standard for Disinfection. Ministry of Health of People's Republic of China, Beijing (in Chinese).

Nagarkar, P.P., Ravetkar, S.D., Watve, M.G., 2001. Oligophilic bacteria as tools to monitor aseptic pharmaceutical production units. *Appl. Environ. Microbiol.*, **67**(3): 1371-1374. [doi:10.1128/AEM.67.3.1371-1374.2001]

Newcombe, D.A., Schuerger, A.C., Benardini, J.N., Dickinson, D., Tanner, R., Venkateswaran, K., 2005. Survival of spacecraft-associated microorganisms under simulated martian UV irradiation. *Appl. Environ. Microbiol.*, **71**(12): 8147-8156. [doi:10.1128/AEM.71.12.8147-8156.2005]

Ogunniyi, T.A.B., Oni, P.O., Juba, A., Asaolu, S.O., Kolawole, D.O., 2000. Disinfectants/antiseptics in the management of guinea worm ulcers in the rural areas. *Acta Tropica*, **74**(1):33-38. [doi:10.1016/S0001-706X(99)00057-1]

Onaolapo, J.A., 1995. Contamination of hospital disinfectants and antiseptics. *Int. Biodeter. Biodegr.*, **36**(3-4):471-472. [doi:10.1016/0964-8305(96)81852-X]

Park, H.D., Noguera, D.R., 2007. Characterization of two ammonia-oxidizing bacteria isolated from reactors operated with low dissolved oxygen concentrations. *J. Appl. Microbiol.*, **102**(5):1401-1417. [doi:10.1111/j.1365-2672.2006.03176.x]

Rosch, P., Harz, M., Schmitt, M., Peschke, K.D., Ronneberger, O., Burkhardt, H., Motzkus, H.W., Lankers, M., Hofer, S., Thiele, H., Popp, J., 2005. Chemotaxonomic identification of single bacteria by micro-Raman spectroscopy: application to clean-room-relevant biological contaminations. *Appl. Environ. Microbiol.*, **71**(3):1626-1637. [doi:10.1128/AEM.71.3.1626-1637.2005]

Schicht, H.H., 1998. New European GMP rules for manufacturing sterile medicinal products. *Cleanrooms Int.*, **2**:17-28.

Shaban, A.M., El-Taweel, G.E., Ali, G.H., 1997. UV ability to inactivate microorganisms combined with factors affecting radiation. *Wat. Sci. Technol.*, **35**(11-12):107-112. [doi:10.1016/S0273-1223(97)00243-6]

Teske, A., Sigalevich, P., Cohen, Y., Muyzer, G., 1996. Molecular identification of bacteria from a coculture by denaturing gradient gel electrophoresis of 16S ribosomal DNA fragments as a tool for isolation in pure cultures. *Appl. Environ. Microbiol.*, **62**(11):4210-4215.

USGSA (US General Services Administration), 1992. Federal Standards 209E: Airborne Particulate Cleanliness Classes in Clean Rooms and Clean Zones. General Services Administration, Washington DC.

Wu, G.F., Zhou, X.P., 2005. Characterization of phosphorus-releasing bacteria in a small eutrophic shallow lake, Eastern China. *Wat. Res.*, **39**(19):4623-4632. [doi:10.1016/j.watres.2005.08.036]