J. Kor. Soc. Cloth. Ind.
Vol. 4, No. 6, pp.557-563(2002)
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A Study on the Antibacterial Activity of Chitosan on the MRSA by the AATCC Test Method 100 and Modified AATCC Test Method 100

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Abstract : Water-soluble chitosan and water-insoluble chitosan with molecular weight of 2,000,000, 500,000, 80,000, and 40,000 with more than 90% of degree of deacetylation were produced to test antibacterial activity of chitosan against a pathogenic bacteria, Methicillin Resistant Staphylococcus aureus(MRSA). the AATCC Test Method 100 and Modified AATCC Test Method 100 were used to evaluate the antibacterial activity of chitosan. Antibacterial activity of chitosan/acetic acid solution was the same when they were tested by two different methods, but those of polyester fabrics treated with chitosan/acetic acid solution were different in different antibacterial test. So several problems were found in the experimental methods. The AATCC Test Method 100 seems that excessive nutrition exists in inoculum solution by quantitative analysis on the basis the result of antibacterial activity on chitosan/acetic acid solution and amount of chitosan attached to the surface of treated fabrics.

Key words : Methicillin Resistant *Staphylococcus aureus* (MRSA), AATCC Test Method 100, Modified AATCC Test Method 100, antibacterial activity, Minimum Inhibitory Concentration (MIC)

INTRODUCTION

Since hospital infection by Staphylococcus aureus and Methicillin Resistant Staphylococcus aureus (MRSA) was reported in early 1990s, 'hospital infection by MRSA' has been significantly issued in the medical industry. And, many research results were presented to prevent MRSA infection in the 12th Japan Environment Infection Society held in 1997. And, According to the survey of antibiotic sensitivity conditions in 1994 by the Ministry of Health & Welfare, Japan, MRSA detection rate of hospitals was nationally average 61%. MRSA, as a variety bacteria of *Staphylococcus aureus, is a varied yellow Staphylococcus* to produce b-lactamase, destructing the structure of b-lactam, an antibiotic Methicillin being spread in the world since it was first reported in U.K in 1961. In addition, as it has resistance against most antibiotics, a countermeasure against the pathogen is being needed in the world.

There are many kinds of insecticides to easily kill pathogenic bacteria. However, considering that a textile goods is the secondary skin, contacting human body every time, characteristics of antibacterial agents used in antibacterial treatment shall be also considered. It is desirable that rather antibacterial agents less harmful to human body even with low bacteriocidal activity than organic antibacterial agents more harmful to human body with more excellent bacteriocidal activity be used. Use of antibacterial agents with such safety promoted use of edible natural high molecular compound, and it enables the third function to fabrics itself as well as bacteriocidal activity.

Chitosan is a representative natural high molecular compound, and its bacteriocidal activity has been studied continuously. As Chitosan has nearly same structure with cellulose and $-NH_2$ with high chemical reactivity in molecular structure, it can receive antibacterial treatment for many textile materials including fabrics.

In this study, experiment with the AATCC Test Method 100 and Modified AATCC Test Method 100 was performed to evaluate antibacterial effect by molecular weight of chitosan/acetic acid solution and that of cotton filter treated with chitosan/acetic acid solution against a pathogen MRSA, and the problems of test methods for antibacterial effect that are being commonly used in the world were verified by analysis of data obtained from a large quantity of experimental results.

MATERIAL AND METHOD

Chitosan

Chitosan used in this study was gained according to following process; a shell was treated with HCl solution to remove calcareous ingredients; the treated shell was heated with NaOH water solution to remove protein ingredients, resulting in gaining Chitin; then the separated Chitin was deacetylated and separated/refined. In result, 5

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Table 1. Characteristics of water insoluble chitosan with controlled molecular weight

Chitosan types	Solubility	Molecular weight	Degree of deacetylation (%)
A+	water insoluble	2,000,000	98.9
A		500,000	97.1
B		150,000	90.4
C		80,000	89.2
D		40,000	87.3

types of water insoluble chitosan were gained. Their whole characteristics are shown in Table 2.

Production of chitosan/acetic acid solution : Acetic acid was used as a solvent, and concentration of acetic acid was made same as that of chitosan but it was mixed between 0.1% and 0.05%, agitated for 24 hrs at room temperature, and then chitosan/acetic acid solution without dissoluble element was obtained. The produced chitosan/acetic acid solution was used within 24 hours after completion of dissolution to minimize possibility of decrease in molucular weight due to cut of molecular chain by action of acetic acid.

As it was confirmed that Minimum Inhibition Concentration(MIC) was 0.05%(5 ppm) as a result of repetitive experiment of Tube Dilution Technique(TDT) against MRSA, concentration was adjusted to 0.025%, 0.05%, 0.075%, and 0.1%.

Chitosan/acetic acid solution treatment of polyester fabrics : The surface of polyester fabrics (100% polyester composed of plain fabrics with 75 dx75 d for yarn number, 210 strands/5 cmx191 strands/5 cm for density of warp and weft, width of 0.11 mm, weight of 72 g/m^3) was treated with chitosan/acetic acid solution by padding on the polyester with 80% of wet pick-up rate, then it was naturally dried at laboratory temperature for 3 days after completion of treatment.

Host and medium

MRSA ATCC 33592 was used as host and Bacto Nutrient Broth(NB) and Nutrient Agar(NA) of Difco were used as medium. NA was used as slant medium for suspension culture of bacteria and plate medium for viable measurement, and NB was used as the lowest growth inhibition concentration test medium and lowest disinfection test medium with x10 system dilution and OD(Optical Density) Method.

Culture method

As MRSA is a commonly aerobic bacteria(Dengrmont and Membre, 1995), for short-term keeping, it was transplanted into NA slant culture and cultured at 37°C for 24 hrs, then was cold kept at less than 5°C, and for long-term keeping of 6-month period, microbial solution cultured in liquid broth medium was put into 30% glycerol and kept at less than -20°C.

For pure culture of bacteria, keeping host 1 loop was taken and transplanted into NB broth culture and cultured in 37°C shaking incubator for 20 hrs, and then this culture solution 1 loop was transplanted into a new NB medium 10 ml and cultured for 18 hrs.

For Cell counting, plate count(colony count) for viable bacteria measurement was used, which requires much time but is very sensitive. At this time, plate culture that formed colony was used through x10 system dilution to be statistically reliable 30~300 colony.

Measurement of antibacterial activity against MRSA

Until now, well-known antibacterial effect test methods are Halo Test Method(AATCC Test Method 90, KS K 0693) and Parallel Streak Test Method(AATCC Test Method 147, KS K 0890) of qualitative method and Bioassay Test(AATCC Test Method 100, KS K 0693), Modified AATCC Test Method 100, Shake Flask Method, Modified Shake Flask Method, and cell counting(JIS L 1902) of quantitative method.

Among those, AATCC Test Method 100 was devised as a suitable method for non-gushing material as chitosan and has been much used by other researchers(Kim, Choi, Im, 1998. Seong, Go, Song, 1998. O, Kim, Choi, 1997). While Modified AATCC Test Method 100 was devised to be applied both to gushing material and non-gushing material. So both the two methods were used in test of polyester fabrics treated with chitosan/acetic acid solution and chitosan.

RESULT AND CONSIDERATION

Measurement of antibacterial activity of chitosan/acetic acid solution

Antibacterial activity of chitosan/acetic acid solution by AATCC Test Method 100 and modified AATCC Test Method 100 : The results of tests for AATCC Method 100 and modified AATCC Method 100 are shown in Table 2. When concentration of chitosan/acetic acid solution was 0.025%, the reduction in bacteria(%) was 99% regardless of molecular weight of chitosan.

As acetic acid was used as a solvent of chitosan, it did not show any trouble in less than 0.05~0.5% of concentration used in this experiment in researching and testing the effect of acetic acid on bacteria. Therefore, effect of acetic acid was neglected.

Table 2. Antibacterial activity of chitosan/acetic acid solution by AATCC Test Method 100 and Modified AATCC Test Method 100.

Test Method	Test Solution		Mean colony number (CFU/ml)	Reduction in bacteria(%)	Remarks
AATCC Test Method 100	0.025%	inoculum size	7.7×10^4		0.1~0.05% concentration of chitosan solution : 99% reduction in bacteria
		control	1225×10^4		
		2,000,000	66		
		500,000	27		
		150,000	97	99	
		80,000	37		
		40,000	83		
Modified AATCC Test Method 100	0.025%	inoculum size	$3.5\sim4.5\times10^4$		
		control	10×10^4		
		2,000,000	2105		
		500,000	166	99	
		150,000	154		
		80,000	1	99.9	
		40,000	0	100	

MIC of Chitosan/acetic acid solution : The lowest concentration of chitosan/acetic acid solution to hinder multiplication of MRSA was represented and MIC to MRSA of chitosan was derived. In the AATCC Method 100 and modified AATCC Method 100, MIC of chitosan were 125 ppm respectively.

Table 3 describes that molecular weight of chitosan does not absolutely affect reduction of multiplication in MRSA. This verifies that, in case of adding chitosan to cultivate MRSA bacteria, chitosan contacts bacteria under the condition that chitosan is dissolved completely in acetic acid solution so that molecular weight of chitosan does not have a great effect on reduction of multiplication in MRSA.

Considering various experimental results to examine antibacterial activity of fabrics treated with chitosan(Seong, Go and Song, 1998. Seong, Kim and Go, 1997. Lee, Nam and Go, 1999. Lee, Nam, Seong and Go, 1998), the researcher expected the molecular weight to affect antibacterial activities against MRSA. Therefore, it seems to be understood that the state of chitosan solidified and attached to the surface of treated fabrics and antibacterial activity of chitosan can have a difference.

Table 3. MIC Value of chitosan/acetic acid solution by antimicrobial activity test method of two types.

Molecular weight of chitosan	AATCC Test Method 100	Modified AATCC Test Method 100
2,000,000	0.025%* (125 ppm)**	0.025% (125 ppm)
500,000		
150,000		
80,000		
40,000		

* % represent concentration of chitosan/acetic acid solution for MRSA

** ( ) represent MIC of chitosan/acetic acid solution

For the chitosan treated on fabrics, change in its contact ability with chitosan and MRSA attached to fabrics as well as degeneration of chitosan can appear by change in processing conditions, and resultingly, the results of experiment to examine antibacterial activity can be changed by each experimentor. Therefore, it is likely that molecualr weight of chitosan affects somewhat antibacterial activity against bacteria.

Measurement of antibacterial activity of fabrics treated with chitosan

Antibacterial activity of polyester fabrics treated with chitosan by the AATCC Test Method 100 : The result of pretesting cotton filter paper coated and processed with chitosan/acetic acid solution, whose concentration was changed into 0.05%, 0.1%, 0.25%, and 0.5%, by the AATCC Test Method 100 did not show regularity. In addition, consistency did not confirmed including forming more colony in the treated fabrics than untreated fabrics frequently. Accordingly, the researcher treated polyester fabrics with chitosan/acetic acid solution and tested antibacterial activity and the results are shown in Table 4.

As shown in Table 4, reduction of multiplication in MRSA reached 95% regardless of molecular weight of chi-

Table 4. Antibacterial activity of the polyester fabrics treated with chitosan/acetic acid solution by AATCC Test Method 100

Test Method	Test condition	Test solution		Mean colony number(CFU/ml)	Reduction in bacteria(%)	Remarks
AATCC Test Method 100	padding on the polyester	0.25%	inoculum size	1×10^4	About 95	0.1% coating concentration of chitosan/acetic acid solution: 99% reduction in bacteria only molecular weight 80,000, 0.5%: all 99% reduction in bacteria
			control	$1\sim2\times10^4$		
			2,000,000	631		
			500,000	924		
			150,000	39		
			80,000	654		
			40,000	16		

tosan in treating with chitosan/acetic acid solution whose concentration is 0.25%. When concentration of chitosan/acetic acid solution reduced to 0.1%, only chitosan with 80,000 of molecular weight showed 99% reduction of multiplication in bacteria. When concentration of chitosan/acetic acid solution increased to 0.5%, antibacterial activity was measured to 99% regardless of molecular weight.

The results of antibacterial activity test did not show consistency in pretesting cotten filter paper by AATCC Test Method 100 and MIC value was measured at 0.25% of concentration of chitosan/acetic acid solution on polyester fabrics. It is assumed that these results mean contact with inoculum solution and finished fabrics is inferior and fiber made of fabrics have hydrophobic property, affecting greatly antibacterial activity. Especially, polyester fabrics are very strong hydrophobic and have low affinity to inoculum solution so that antibacterial activity can be reduced rapidly.

Antibacterial activity of the polyester fabrics treated with chitosan by modified AATCC test method 100 : Table 5 shows the results of antibacterial activity test of polyester fabrics treated with chitosan/acetic acid solution by modified AATCC Test Method 100.

When concentration of chitosan/acetic acid solution treated on polyster fabrics was over 0.025%, reduction of growth in MRSA reached 100% regardless of molecular weight.

As shown in Table 5, growth in MRSA was reduced under 0.25% or higher of concentration of chitosan/acetic acid solution when antibacterial activity of polyester fabrics treated with chitosan was measured by the AATCC Test Method 100, whereas growth in MRSA was reduced under 0.025% or higher of concentration of chitosan/acetic acid solution when antibacterial activity was measured by the modified AATCC Test Method 100. It was shown that a difference in antibacterial activity was high between AATCC Test Method 100 and modified AATCC Test Method 100.

The antibacterial activity of fabrics treated with chitosan was reduced to 1/10 by the AATCC Test Method 100 rather than modified AATCC Test Method 100. The reason seems that sufficient nutrition exists in inoculum solution for the AATCC Test Method 100.

In other words, the fundamental difference between AATCC Test Method 100 and modified AATCC Test Method 100 was just amount of nutrition in inoculum solution.

MIC of MRSA in treating fabrics with chitosan/acetic acid solution : Table 6 showed the minimum concentration of chitosan/acetic acid solution for reducing multiplication in MRSA when fixing the wet pick up to 80% and treating polyester fabrics with chitosan/acetic

Table 5. Antibacterial activity of the polyester fabrics treated with chitosan/acetic acid solution by modified AATCC Test Method 100.

Test condition	Test solution		Mean colony number (CFU/ml)	Reduction in bacteria(%)	Remarks
padding on the polyester	0.025%	inoculum size	$1\sim1.5\times10^4$	99	0.1~0.05% coating concentration of chitosan/acetic acid solution : 99% reduction in bacteria
		control	$1.5\sim2\times10^4$		
		2,000,000	153		
		500,000	126		
		150,000	286		
		80,000	272		
		40,000	269		
		F(oligo)	207		

Table 6. MIC of MRSA on the chitosan/acetic acid solution and the filters treated with chitosan/acetic acid solution.

Chitosan type (molecular weight)	AATCC Test Method 100	Modified AATCC Test Method 100
2,000,000		
500,000	0.25%	0.025%
150,000	(250 ppm)	(25 ppm)*
80,000	(PET fabrics)	(PET fabrics)
40,000		

*( ) value is MIC of chitosan/acetic acid solution

acid solution. Also, Table 6 described the minimum concentration of chitosan/acetic acid solution for reducing multiplication in MRSA as well as the minimum concentration of chitosan for reducing multiplication in MRSA by being solidified and attached to the surface of fabrics.

In Table 6, it was shown that the minimum concentration of chitosan/acetic acid solution for reducing growth of MRSA has been changed by antibacterial testing methods. By calculating amount of chitosan attached to the surface of fabrics treated with chitosan, MIC value against MRSA can be also calculated as follows;

In the AATCC Test Method 100, the growth of MRSA is reduced when fabrics was treated with 0.25% of chitosan/acetic acid solution. Therefore, weight of chitosan solidified and attached to the surface of treated fabrics of 0.2 g is 0.2×(0.25/100)=5×10-4 g. And, as 0.2 ml of inoculum is added to 0.2 g of fabrics treated with chitosan, concentration of chitosan in inoculum is calculated to 5×10-4 g chitosan/0.2 ml i.e., 2.5×10-3 g/ml, and resultingly MIC of the solidified chitosan is 2500 ppm.

Accordingly, in the AATCC Test Method 100, antibacterial activity of chitosan attached to fabrics treated with chitosan reduced below 1/20 approximately compared to antibacterial activity of chitosan.

But, the above AATCC Test Method 100 has multiple problems; nutrition in inoculum is excessive compared to cultivating time; amount of bacterial solution is also large when inoculating 0.2 ml of inoculum to 0.2 g of treated fabrics; inoculum solution can be attached in inoculation as pipette does not reach testing tube for 30 ml to the end; and other tools such as loop should be applied to soak fabric inoculum. Therefore, it is decided that individual difference in testing treatment is large and antibacterial activity is not reliable.

In the modified AATCC Test Method 100, MIC is reduced to 1/10 compared to the AATCC Test Method 100. The reason is that nutrition in the inoculum solution is reduced to 1/15 unlike the ATCC Test Method 100.

But, in the modified AATCC Test Method 100, MIC of chitosan was 125 ppm while MIC of chitosan solidified and attached to the surface of treated fabrics was 250 ppm. It cannot be understood that MIC of chitosan attached to the surface of treated fabrics increased just twice unlike the MIC of chitosan as compared to antibacterial activity of chitosan in studies until now.

It has poor grounds of arguments that a difference of antibacterial activity between chitoasan/acetic acid solution dissolved completely in measuring antibacterial activity of chitosan and chitosan - solidified and attached to the treated fabrics - with difficulties in expressing its antibacterial activity is insignificant just twice.

In measuring antibacterial activity of chitosan, both AATCC Test Method 100 and modified AATCC Test Method 100 have the same MIC value (125 ppm) (Table 3).

In the above testing methods, the reason why MIC values are identical though different nutrition supplying conditions is that chitosan exists under dissolved conditions to show the maximum antibacterial activity, and chitosan expresses antibacterial activity not to be affected by the difference in addition of nutrition at the same time.

On the other hand, in fabrics treated with chitosan, antibacterial activity by bacterial solidified and attached to the surface of fabrics is not smooth, antibacterial activity is only to reduce rapidly compared to antibacterial activity of chitosan, and MIC value increases also significantly.

In the modified AATCC Test Method 100, MIC of fabrics treated with chitosan increases just twice unlike MIC of chitosan and increase of MIC value is too low to be reliable as compared to the AATCC Test Method 100. Though the modified AATCC Test Method 100 has more objective and excellent reliability than the AATCC Test Method 100, it has still problems proposed in the AATCC Test Method except reduction of nutrition to 1/15 and the reliability is poor. And, it had different from wearing conditions as antibacterial activity was measured by dropping and permeating inoculum to the treated fabrics.

Strength and weakness of AATCC test method for antibacterial treated Fabrics

As shown in the aforesaid, in measuring antibacterial activity of antibacterial-treated filter, many problems were derived from the antibacterial test methods including inconsistency of the results in testing cotton filter paper by the AATCC Test Method 100 and large difference in antibacterial activity between the AATCC Test Method 100 and modified AATCC Test Method 100. The derived problems were divided into strength and weakness of each test method, then compared as shown in Table 8 and 9.

Table 8. Strength and weakness of AATCC Test Method.

Strength	①It gives conditions for microorganisms to grow. (culture temperature, culture time, addition of nutrition) →Oxygen is insufficient compared to in wearing condition. ② Considering number of skin flora is about 1×10^8 cell/ml in the highly sweaty region such as armpit, antibacterial test is performed in similar conditions to the particular region of human body.
Weakness	① Nutrition included in the inoculated bacterial solution is different from the actual environment for wearing condition, compared to amount of nutrition transferred from the outside or nutrition separated from skin and stuck on fabrics. ② It cannot be clearly confident if bacteria included in the inoculated bacterial solution are those that correspond to the logarithmic growth phase in life cycle. ③ Number of bacteria included in the inoculated bacterial solution is defined as 1~2×105 cell/ml. But, it cannot be correctly measured and correct value cannot be confirmed. ④ Number of bacteria included in the inoculated bacterial solution is defined as 1~2×105 cell/ml. But, it cannot be correctly measured and number of early bacteria can be different by inspectors, so possibility that difference in antibacterial performance may occur is high. ⑤ In inoculation of a fiber and inoculated bacterial solution, bath ratio is defined as 1:1. But, amount of inoculated bacterial solution is more than that of fiber mostly, so bacterial solution remains after fiber is soaked completely. ⑥ Testing tube for 30 ml used in test is not inoculated completely in the ratio of 1:1 with test fabrics as end of pipette is not contacted in inoculation, so bacterial solution can lean to one side or outside test fabrics. ⑦ Time goes by until the inoculation time after making a culture medium for the inoculated bacterial solution, and resultingly additional multiplication of bacteria may cause problems. ⑧ Inoculation and culture of fiber and inoculated bacterial solution are performed in an airtight vessel. For aerobic bacteria, exclusion of oxygen by airtight gives stress. And, exclusion of oxygen is greatly different from the actual environment for wearing condition. ⑨ Inoculated bacteria stuck on test fabrics cannot be completely extracted with physiological saline solution. ⑩ Waterdrop phenomenon may appear in vial included test fabrics and bacterial solution due to wet sterilization. ⑪ Elution-type bacteria results in beneficial effects, while antibacterial test results for non-elution type bacteria cannot be confident in principle. ⑫ Water repellence of test fabrics has great influence on contact condition of inoculated bacterial solution and test fabrics. Therefore, fiber type composing test fabrics has great influence on antibacterial activity.

Table 9. Strength and weakness of Modified AATCC Test Method 100

Strength	Nutrition of inoculated bacterial solution proposed in the AATCC Test Method 100 was adjusted to be similar to the amount stuck on fabrics.
Weakness	Except that some of item ① among weaknesses proposed in the above AATCC 100 is settled, most weaknesses proposed in the AATCC Test Method 100 remain as it is.

CONCLUSION

To examine antibacterial activity of chitosan against MRSA of a pathogenic bacteria, chitosan/acetic acid solution was treated with 5 types of water-insoluble chitosan with up to 90% of deacetylation and 2,000,000/500,000/150,000/80,000/40,000 of molecular weight on polyester fabrics, then antibacterial activity was measured. By applying the AATCC Test Method 100 and modified AATCC Test Method 100, results were follows.

1. With repect to antibacterial activity of chitosan/acetic acid solution, MIC value was 125 ppm both AATCC Test Method 100 and modified AATCC Test Method 100.

2. In the AATCC Test Method 100, antibacterial activity of chitosan attached to fabrics treated with chitosan/acetic acid solution reduced below 1/20 approximately compared to antibacterial activity of chitosan/acetic acid solution. In the AATCC Test Method 100, MIC value reduced to 1/10 compared to the modified AATCC Test Method 100. These results seem that test methods have some problems.

3. AATCC Test Method 100 does not satisfy well enough the temperature and nutritional conditions for the cultivation, nor meet with the requirements for simulating the actual wearing conditions. Even the modified AATCC Test Method 100, which simply reduced the amount of nutrients down to 1/15 of the requisition of AATCC Test Method 100, does not fully satisfy the requirements. It could well be said that there is not

much significant methodolgical improvement over the original AATCC Test Method 100 based on the result that there is a noticeable difference between the antibacterial activity of the chitosan itself and that of the chitosan-treated fabrics. It is thereby suggested that there is a prime need for the further development of a novel testing method for determining the antibacterial activity of the antibacterial agent itself and that of the treated fabrics accurately and easily.

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(Received October 14, 2002)