THE FACTS
ISSUE 3 • 2007
www.healthandhygiene.co.za
F10 PRODUCTS







MINIMUM INHIBITORY CONCENTRATION (MIC) AND ZONE OF INHIBITION DETERMINATION METHODS TO BE USED WHEN TESTING F10SC DISINFECTANTS

Neil Forbes BVetMed *Dip ECAMS FRCVS,* **Elize Lloyd** *BSc Hons Microbiology*

Summary

F10 Super Concentrate Disinfectant (F10SC) is a quaternary ammonium and biguanidine compound based disinfectant. Independent tests have shown it to be effective against bacteria, fungi, viruses and spores (bacterial and fungal spores). It had been reported that when using the sensitivity/resistance zone of inhibition test method on bacterial isolates that inconsistent results occur. A study[1] was initiated to determine the most appropriate laboratory method to be used for evaluation of F10SC when using zone of inhibition test methods using commercially available susceptibility dics of 10µg Gentamicin and 5 µg Enrofloxacin as controls.

In 1036 readings all eight organisms tested were sensitive to F10SC disinfectant in a dilution of 1/250 in broth and complete visual inhibition was observed at this and much lower levels. The more resistant organisms, for example *Pseudomonas aeruginosa* and *Escherichia coli*, produced small inhibition zones on agar at a concentration equal to a 1/250 dilution, but were still completely inhibited by 1/1000 and 1/4000 dilutions of F10SC in broth respectively. The more sensitive organisms, for example *Staphylococcus aureus*, produced large inhibition zones on agar at a concentration equal to a 1/250 dilution of the product and were completely inhibited in broth by dilutions as low as 1/16 000. A total of 146 readings were taken of each of the Gentamicin and Enrofloxacin controls.

However the study did show that this type of test must be carried out within strict parameters otherwise inconsistent and misleading results would be obtained.

Introduction

The study was conducted with the following objectives :

- To investigate an agar disc diffusion zone of inhibition test method for evaluating the susceptibility of aerobic and facultative anaerobic bacteria against F10SC.
- To compare zone inhibition results over a range of four to six different concentrations of F10SC on two different types of agar normally used for susceptibility testing i.e. Mueller Hinton agar (supplier Merck) and Iso-Sensitest Agar (Oxoid) (supplier C. A. Milsch).
- To compare the sensitivity or resistance of the eight test organisms to commercially available susceptibility discs gentamicin 10µg and Enrofloxacin 5µg on two different types of agar.
- To establish the MIC values of the agent against a number of commonly occurring bacterial pathogens, these included known ATCC strains, local isolates and avian pathogens.

The Study

Test organisms

The following organisms were used:

Pseudomonas aeruginosa	ATCC 27853
Escherichia coli	ATCC 25922
Staphylococcus aureus	ATCC 25923
Klebsiella pneumoniae	ATCC 10031
Staphylococcus aureus MRSA	Local Isolate supplied by: H F Verwoerd Hospital 3/1995 B/N 177/6
Pasteurella multocida	Local Avian Isolate supplied by: Veterinary Faculty Onderstepoort
Enterococcus faecalis	ATCC 29212
Salmonella choleraesuis serotype *typhimurium*	ATCC 13311

Preparation of bacterial suspensions

According to NCCLS guidelines[4] the direct colony suspension method was selected.

The cultures were grown on non selective agar (nutrient agar slopes) for 16 - 18 hours at 35°C (± 2°C) and growth was re-suspended with sterile 0,85 % saline. The concentrated suspensions obtained were diluted with sterile 0,85 % saline to match the turbidity of a 0,5 McFarland standard (approximately 70 - 75 %T at 600 nm). The resulting bacterial suspensions contained approximately 1,5 x 10^8 cfu/ml .

Macro-broth dilution MIC determination

Broth dilution MIC determination was performed in accordance with the guidelines as described by the NCCLS[4].

Mueller-Hinton broth (pH 7,2 - 7,4) supplied by Merck. The broth was prepared and sterilized according to the manufacturer's instructions. The cation concentration of the broth was not adjusted and the broth was used "as is".

Eleven tubes were prepared in duplicate for each test organism starting with a 1/125 dilution of F10SC (0,8%) made directly in Mueller-Hinton broth. Tube 1 contained 1 ml of this solution. Sterile Mueller-Hinton broth (1 ml) was put into tubes 2 - 11 and the starting concentration of 1/125 was diluted 1:1 with the sterile broth in tube 2, resulting in a concentration of 0,4% or a dilution of 1/250 in tube 2. Further serial dilutions were done in the same way up to tube number 9 covering a range of dilutions from 1/125 to 1/32 000. Tube 10 contained inoculated broth and served as a positive control. Tube 11 contained uninoculated broth and served as a negative control. Both tubes 10 and 11 contained no anti microbial agents. All tubes contained 1 ml of Mueller-Hinton broth.

The bacterial suspensions obtained with the direct colony suspension method contained approximately $1,5 \times 10^8$ cfu/ml. These suspensions were further diluted 2 ml in a total of 10 ml with sterile saline to give an approximate count of 3×10^7 cfu/ml. Each tube containing a total volume of 1 ml broth (except the negative control tube) was inoculated with 10 µl (0,01 ml) of this diluted organism suspension resulting in an approximate count of 3×10^5 cfu/ml or per tube.

Total counts were done (serial dilutions) on plate count agar to confirm the viable number of organisms that were present in the test suspensions.

Tubes were incubated 16 - 20 hours at 35°C. The MIC value obtained is interpreted as the concentration of the anti microbial agent, contained in the first tube in the series, that inhibits visible growth of the test organism.

Agar disc-diffusion zone of inhibition determination.

The Kirby Bauer method of agar disc-diffusion zone of inhibition determination as described by Koneman et al[3] was used.

Mueller-Hinton agar (pH 7,2 7,4) supplied by Merck. The agar was prepared and sterilized according to the manufacturer's instructions.
(± 25 ml was poured into 90 mm Petri dishes. Agar thickness approximately 4 mm.)

Oxoid Iso-Sensitest agar supplied by C A Milsch. The agar was prepared and sterilized according to the manufacturer's instructions. (± 25 ml was poured into 90 mm Petri dishes. Agar thickness approximately 4 mm.)

Preparation of discs and F10SC stock solutions

To achieve the same levels of active ingredients present in 1 ml of a specific broth dilution of F10SC on discs containing a volume not exceeding 10 µl (0,01 ml), it was necessary to prepare stock solutions of F10SC:
For example:

Desired disc content:
0,4 % F10SC (a dilution of 1/250 of F10SC is equal to 0,4 % of F10SC).

$$\frac{\text{Desired disc content: 0,4 \% F10SC}}{\text{Amount delivered to disc (0,01 ml)}} = \text{concentration of stock solution (40 \%)}$$

Thus 0,01 ml of a 40 % solution on a disc contained the same amount of active ingredients as 1 ml of a 0,4 % solution of F10SC. Stock solutions ranging from 40 % to 1,25 % were prepared and used on the discs. These represented 1 ml broth dilutions of F10SC ranging from 1/250 to 1/8000.

In previous tests 20 µl of less concentrated stock solutions were used on the discs with poor results. Volumes in excess of 10 µl cause distortion of the inhibition zones because of the limited ability of the discs to fully absorb such volumes.

Preparation of Petri dishes

Sterile blank discs (Mast) 6,5 mm in diameter of were obtained from Davies Diagnostics.
Approximately 130 plates each of Mueller-Hinton agar and Iso-Sensitest agar were used for the tests. Tests at all levels of F10SC were done on duplicate plates for each type of agar. An average of six discs were used over two pates for each F10SC dilution.

All agar plates were inoculated with bacterial suspensions prepared according to the direct colony suspension method as previously described.

Plates at room temperature were streaked 3 times with cotton swabs dipped into the suspension over the entire agar surface, rotating the plates approximately 60° to ensure an even distribution of the test organism. Excessive moisture was avoided.

Plates were left 3-5 minutes to dry. Blank discs were put onto the inoculated surfaces of the Mueller-Hinton and Iso-Sensitest agar plates using sterile forceps. Approximately three discs per 90 mm plate were used. Gentle pressure was applied with sterile forceps to ensure complete contact of discs with agar. Oxoid gentamicin 10µg and Enrofloxacin 5µg reference discs were also applied to both types of inoculated agar.

A micro pipette (Eppendorff pipette) was used to drip 10 µl discs of the various stock solutions of F10SC onto the discs.

Plates were not inverted and were incubated within 15 minutes of disc application for 16 - 18 hours at 35ºC (± 2ºC). All testing was done in duplicate and total counts of all the bacterial suspensions were done.

Quality Control of media

Three ATCC reference strains of test organisms were used for the quality control of the Mueller-Hinton agar and broth:

Pseudomonas aeruginosa	ATCC 27853
Escherichia coli	ATCC 25922
Staphylococcus aureus	ATCC 25923

Mueller-Hinton agar: All three organisms were used. The agar depth was controlled at approximately 4 mm to minimize variability in zone sizes. Commercially available Oxoid susceptibility discs gentamicin 10µg were put on Mueller-Hinton plates used for the testing of F10SC. The average inhibition zone sizes obtained for gentamicin were well within the stated acceptable ranges for each specific reference organism as indicated by the NCCLS[4].

Mueller-Hinton broth: *Pseudomonas aeruginosa* ATCC 27853, and *Escherichia coli* ATCC 25922 were used for the quality control of the broth. SABS could not supply the *Staphylococcus aureus* ATCC 29213 reference strain suggested by the NCCLS[4] for the testing of Mueller-Hinton broth. The use of *Pseudomonas aeruginosa* ATCC 27853 and *Escherichia coli* ATCC 25922 for quality control purposes was considered sufficient to validate the results. Serial dilutions were prepared in Mueller-Hinton broth containing gentamicin ranging from 10 - 0,078 µg/ml. MIC values observed for both organisms were well within the acceptable ranges as specified by the NCCLS[4]

Positive controls: Sterile Mueller-Hinton broth, Mueller-Hinton agar plates and Iso-Sensitest agar plates were inoculated with the test organism suspensions used for the relevant tests and good growth was observed after 16-20 hours incubation.

Negative controls: Sterile Mueller-Hinton broth, Mueller-Hinton agar plates and Iso-Sensitest agar plates were incubated under the same conditions as the rest of the test tubes and plates to confirm sterility.

RESULTS

MIC values in Mueller-Hinton broth

Average result of both tubes tested for each organism indicated as one result only where results were the same. Both results indicated where differences between the duplicate test results were observed.

Table 1

Tube number	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10** Negative Control	**11** Positive Control
Concentration of F10SC	0,8%	0,4%	0,2%	0,1%	0,05%	0,025%	0,0125%	0,00625%	0,0031%		
Dilution of F10SC	1/125	1/250	1/500	1/1000	1/2000	1/4000	1/8000	1/16000	1/32000		
Pseudomonas aeruginosa ATCC 27853	-	-	-	-	+++	+++	+++	+++	+++	-	+++
Escherichia coli ATCC 25922	-	-	-	-	-	-	++	+++	+++	-	+++
Staphylococcus aureus *ATCC 25923*	-	-	-	-	-	-	-	-	-/+	-	+++
Klebsiella pneumoniae ATCC 10031	-	-	-	-	-	-	-/+	++	+++	-	+++
Staphylococcus aureus *MRSA* Local isolate	-	-	-	-	-	-	-	-	++	-	+++
Pasteurella multocida Local isolate	-	-	-	-	-	-	-	-	+++	-	+++
Enterococcus faecalis ATCC 29212	-	-	-	-	-	-	-	-	++	-	++
Salmonella choleraesuis *Serotype* typhimurium ATCC 13311	-	-	-	-	-	-	-	+++	+++	-	+++

+++ indicated good visible growth of the test organism
- indicated the absence of visible growth of the test organism

The F10SC MIC values Figure A

Test organism:	Concentration of F10SC (dilution) that resulted in complete visual inhibition of the test organism = MIC value
Pseudomonas aeruginosa ATCC27853	A dilution of 1/1000 or 0,1 % F10SC
Escherichia coli ATCC25922	A dilution of 1/4000 or 0,025 % F10SC
Staphylococcus aureus ATCC 25923	A dilution of 1/16 000 or 0,00625% F10SC
Klebsiella pneumoniae ATCC 10031	A dilution of 1/4000 or 0,025 % F10SC
Staphylococcus aureus *MRSA* Local isolate	A dilution of 1/16 000 or 0,00625% F10SC
Pasteurella multocida Local isolate	A dilution of 1/16 000 or 0,00625% F10SC
Enterococcus faecalis ATCC29212	A dilution of 1/16 000 or 0,00625% F10SC
Salmonella choleraesuis *Serotype* typhimurium *ATCC 13311*	A dilution of 1/8000 or 0,0125 % F10SC

Repeatability of results: The values obtained for *Pseudomonas aeruginosa* ATCC 27853, *Escherichia coli* ATCC 25922 and *Staphylococcus aureus* ATCC 25923 were the same as obtained in previous tests under the same conditions[2].

Diffusion susceptibility results

All the organisms tested were sensitive to F10SC in a dilution of 1/250 in broth and complete visual inhibition was observed at this and much lower levels. The more resistant organisms, for example *Pseudomonas aeruginosa* and *Escherichia coli* , produced small inhibition zones on agar at a concentration equal to a 1/250 dilution, but were still completely inhibited by 1/1000 and 1/4000 dilutions of F10SC in broth respectively. The more sensitive organisms, for example *Staphylococcus aureus*, produced large inhibition zones on agar at a concentration equal to a 1/250 dilution of the product and were completely inhibited in broth by dilutions as low as 1/16 000.

The effect of F10SC concentrations on eight different test organisms was measured. Average inhibition zone sizes were noted in mm. (An average of 6 discs were used for each F10SC dilution over two plates)

Zone measurements

The effect of F10SC concentrations on eight different test organisms. Inhibition zone measurements in mm. (± 6 discs per F10SC dilution over two plates.

Table 2 - using Mueller-Hinton Agar.

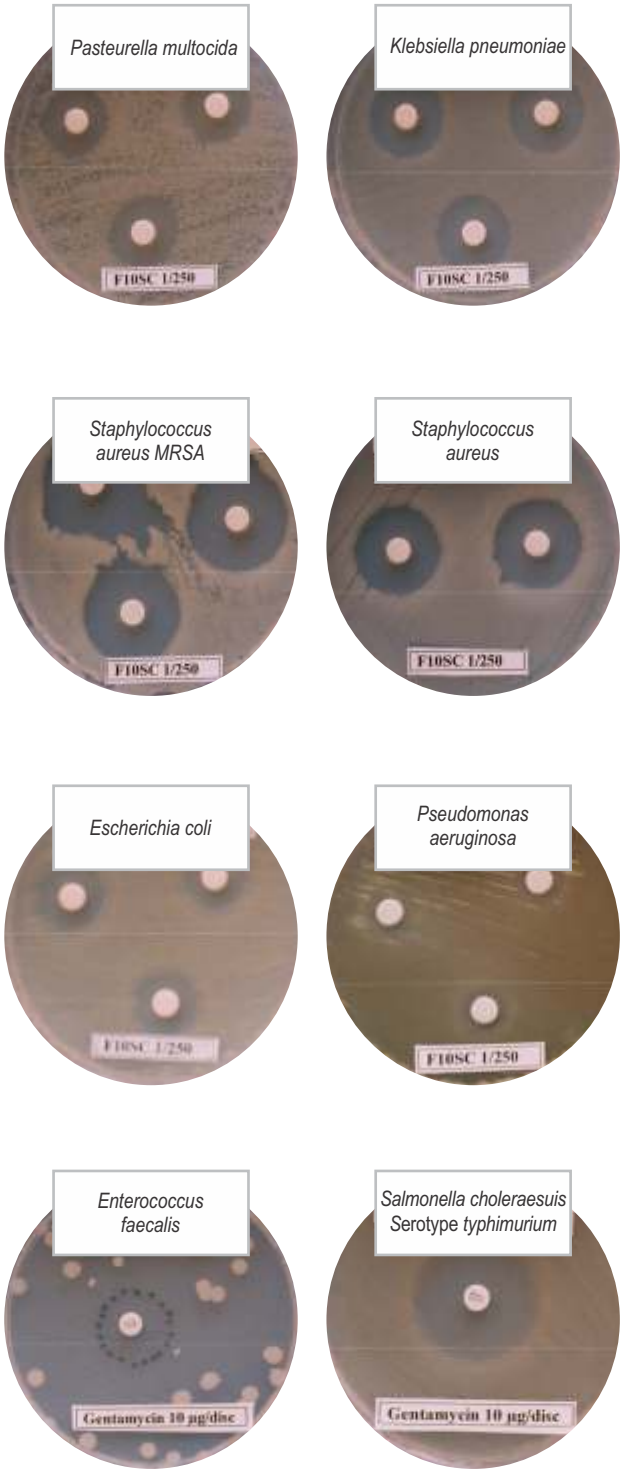
	Dilution of F10SC					
Dilution	1/250	1/500	1/1000	1/2000	1/4000	1/8000
%F10SC (1ml/100ml) **on discs containing 10µl of stock solution**	0,4%	0,2%	0,1%	0,05%	0,025%	0,0125%
F10SC stock solution used for the disc preparation	40%	20%	10%	5%	2,5%	1.25%
Pseudomonas aeruginosa ATCC 27853	9,7	9,4	9,1	8,8	8,6	Not tested
Escherichia coli ATCC 25922	16,7	15,1	13,9	12,0	11,2	Not tested
Staphylococcus aureus *ATCC 25923*	23,7	22,3	20,6	17,6 distortion	15,9 distortion	14,9
Klebsiella pneumoniae ATCC 10031	21,7	20,6	19,2	17,8	15,5	14,4
Staphylococcus aureus *MRSA* Local isolate	23,3	23,9	21,3	20,6	16,5	12,6
Pasteurella multocida Local isolate	19,9	17,4	15,3	13,5	Not tested	Not tested
Enterococcus faecalis ATCC 29212	23,0	22,3	19,1	18,2	16,1	13,3
Salmonella choleraesuis *Serotype* typhimurium ATCC 13311	17,7	16,2	13,1	11,6	Not tested	Not tested

Table 3 - Iso-Sensitest Agar

	Dilution of F10SC					
Dilution	1/250	1/500	1/1000	1/2000	1/4000	1/8000
%F10SC (1ml/100ml) **on discs containing 10µl of stock solution**	0,4%	0,2%	0,1%	0,05%	0,025%	0,0125%
F10SC stock solution used for the disc preparation	40%	20%	10%	5%	2,5%	1.25%
Pseudomonas aeruginosa ATCC 27853	10,1	9,5	9,5	9,3	8,9	Not tested
Escherichia coli ATCC 25922	15,6	14,4	12,9	11,0	10,4	Not tested
Staphylococcus aureus *ATCC 25923*	Distortion Severe overlapping	Distortion Severe overlapping	Distortion Severe overlapping	Distortion Severe overlapping	15,6 Distortion overlapping	13,6 Distortion overlapping
Klebsiella pneumoniae ATCC 10031	Distortion Severe overlapping	21,1 Distortion overlapping	19,7 Distortion overlapping	16,1 Distortion overlapping	No Result Distortion overlapping	16,6 Distortion overlapping
Staphylococcus aureus *MRSA* Local isolate	23,3 Distortion	No Result Severe overlapping	Severe overlapping	20,7 Distortion overlapping	21,2	16,7
Pasteurella multocida Local isolate	17,9	15,6	14,3	12,5	Not tested	Not tested
Enterococcus faecalis ATCC 29212	19,8	18,7	17,4	15,9	14,5	13,0
Salmonella choleraesuis *Serotype* typhimurium ATCC 13311	17,2	16,1	14,4	12,3	Not tested	Not tested

F10®
PRODUCTS

Mueller-Hinton Agar



Iso-Sensitest Agar

Conclusions

1) Good growth of all the test organisms was observed on both types of test media. However the final diluted bacterial suspension applied to the plates must be controlled to give counts equal to 0,5 McFarland Standard of approximately 1,5 $\log^8$ cfu/ml in order that the bacterial load is within the capacity of the antimicrobial solution on the disc.

2) Inhibition zones of Gram positive and negative test organisms on Mueller-Hinton agar were in general clear and well defined with very little distortion, even at very high concentrations of F10SC. However the thickness of the agar must be approximately 4mm and placed on a levelled surface.

3) On Iso-Sensitest agar plates a problem was observed with some of the test organisms when F10SC was tested in high concentrations. Severe distortion and overlapping of inhibition zones occurred on plates inoculated with *Staphylococcus aureus, Staphylococcus aureus* MRSA and *Klebsiella pneumoniae*. Discs containing F10SC in concentrations of 1/250; 1/500; 1/1000 and 1/2000 cannot be used for the susceptibility testing of these organisms on Iso-Sensitest agar. Only when the concentration of F10SC was reduced equivalent to 1/4000 and 1/8000 were the zones less distorted and could be measured.

4) It is recommended that Mueller-Hinton agar be used when carrying out susceptibility test with F10SC.

5) Volumes in excess of 10 µl cause distortion of the inhibition zones because of the limited ability of the discs to fully absorb such volumes.

6) In this study only one isolate of a specific species was examined. Because of intra species variation it is currently not possible to establish zone diameter limits for resistant, intermediate and susceptible isolates within a group. The Proposed Agar Diffusion Method for use with F10SC[4] can be used to screen as many isolates of a specific species to establish zone diameter limits for isolates considered to be resistant

References

1. *SABS report No. 2570437/1936/Y64427*
2. *SABS report No. 7218/2547288/1317/Y59521*
3. *Koneman ,E.W. Editor: Colour atlas and textbook of Diagnostic Microbiology, 5th edition. Lippincott Williams &Wilkins, Maryland.*
4. *National Committee for Clinical Laboratory Standards. Performance standards for antimicrobial susceptibility testing; Supplement 1. National Committee for Clinical Laboratory Standards, Wayne, Pa*

F10 PRODUCTS LTD
Unit 7, Windmill Road, Loughborough, LE11 1RA, UK
Freephone: 0800 014 8803 • Fax: 01509 265777
Email: orders@f10products.co.uk • info@f10products.co.uk
www.f10products.co.uk

Manufacturer of F10 Products:
Health and Hygiene (Pty) Ltd
P.O. Box 906, Florida Hills, 1716, South Africa
Tel: +27 11 474 1668 • Fax: +27 11 474 1670
www.healthandhygiene.co.za • www.f10products.co.za