Indian Journal of Applied Microbiology
ISSN (Online): 2454-289X, ISSN (Print): 2249-8400

Volume 23 Number 2
July - September 2021, pp. 11-29

10.46798/ijam.2021.v23i02.2

Time to relook at formulations recommended for hand sanitizers formulations - An in vitro study

Manan Suryavanshi[1], Neelam Sachdeva[2], Jiten Jaipuria[3], Vandana Bhushan[2], Kavita Sharma[2]

[1]IBDP Grade 12, Shiv Nadar School, Pahari Road, Block E, DLF Phase 1, Sector 26A, Gurugram, Haryana 122011

[2]Department of Microbiology, Rajiv Gandhi Cancer Institute and Research Centre, New Delhi-110085, India.

[3]Department of Uro-oncology, Rajiv Gandhi Cancer Institute and Research Centre, New Delhi-110085, India

Abstract

Purpose: The world health organization (WHO) in 2009 in their consensus recommendation on hand hygiene has suggested two formulations of hand sanitizers which are also the basis of main components of most commercial and medical grade hand sanitizers today. We evaluated the in vitro antimicrobial efficacy of ten different hand sanitizers (seven commercial including herbal (sanitizer 1, 2, 3, 4, 5, 9, and 10) and three of medical grade (sanitizers 6, 7 and 8). **Method**: The efficacy of hand sanitizers was checked against five ATCC strains: *Staphylococcus aureus*, *Staphylococcus hemolyticus*, *Pseudomonas aeruginosa*, *Escherichia coli*, and *Enterococcus faecalis*. Experiment was performed in three parts. The first part was performed in triplicate to see the zone of inhibition for each sanitizer. The second part was performed to see the duration of action of each hand sanitizer and third part (in triplicates) was performed to see the efficacy of active components individually (alcohol and disinfectant in different dilutions). **Results:** Sanitizers with ethanol and chlorhexidine as main ingredients (6 and 8) showed zone of inhibition for all tested gram positive and negative bacteria. Sanitizer 7 (propanol and mecetronium ethyl sulphate as main components) showed zone of inhibition for all tested bacteria except *Pseudomonas aeruginosa*. Other hand sanitizers did not show any zone of inhibition after incubation for 24 hours at 37^{o}C. For second part hand sanitizer 6inhibited growth for all bacteria at all-time points (15, 30, 45 &60 seconds) and

*Author for Correspondence. E-mail: sachdevaneelam@gmail.com

Sanitizer 8(ethanol and chlorhexidine as main components) showed growth inhibition only after 15seconds. Other hand sanitizers did not show any growth inhibition. For the third part, all dilutions of ethanol and propanol (60%, 70% and 80%) were unable to inhibit growth of any ATCC strain. Disinfectant 2.5v/v chlorhexidine was able to inhibit all five bacteria.0.2 gm mecetronium ethyl sulphate showed inhibition for all except *Pseudomonas aeruginosa*. **Conclusion:** Hand sanitizers with alcohol only as their main ingredient were unable to inhibit growth of ATCC strains. Hand sanitizers with both alcohol and disinfectant performed better .These findings preludes for further in vivo studies to validate 2009-WHO hand sanitizer preparations and suggest modifications.

Keywords: Hand Sanitizer, WHO, alcohol, ethanol, chlorhexidine, mecetronium ethyl sulphate

Introduction

The skin serves as a protective barrier against many harmful microorganisms. It harbours many useful bacteria like *Staphylococcus epidermis*, *Propionibacterium spp.*, *Corynebacterium spp.*, *Micrococcus spp.* and *Staphylococcus aureus*. These bacteria compete with pathogenic bacteria and prevent their colonization. These good bacteria may be lost by frequent hand washing or use of topical antibiotics [1]. Normally at a given time there are about 10000 organisms per cm^2 of normal skin [2]. Hand bacteria are the common cause of cold, fever, cough and diarrhoea [3]. Thus hand hygiene plays a very crucial role to maintain good health by preventing respiratory, skin and gastrointestinal infections.

Hand washing with soap has been known to reduce infection. Some studies claim that even after hand washing majority of bacteria were not removed in individuals who did not follow proper hand washing steps [4]. Also natural skin oils are removed resulting in cracked skin which could provide a portal for entry of microorganisms[5]. Based on these limitations the concept of hand sanitizers was introduced.

Semmelweis in 1846 was first to start the concept of hand hygiene using chlorinated lime [6].Initially this concept was only for infection control. In 2002 Centre for Disease Control and Prevention (CDC) issued a guideline stating that alcohol based hand rubs which are now called sanitizers could be used as a routine for decontaminating hands [7]. These hand sanitizers decrease infection and their antimicrobial activity is very fast. There is no added need for water or hand drying which were source of contamination. Some studies have shown that hand sanitizers have outperformed hand washing with soap [8]. The World Health Organization in 2009 in their consensus recommendation on hand hygiene in health care workers recommended that alcohol-based hand rubs were a means for fast and effective inactivation of a wide range of potentially harmful microorganisms present on hands. They have suggested two formulations keeping into account cost and microbiological efficacy [9].

WHO also recommends using reagents that are of pharmacopeial quality and refrain from use of technical grade products. Not only the composition but the method of use and duration of use is also elaborated by WHO [9].

There are two categories of hand sanitizer: Alcohol based and alcohol free [10]. The alcohol based sanitizers have isopropanol, n-propanol or ethanol as their active ingredients [11]. On the other hand alcohol-free hand sanitizers use antiseptics for antimicrobial properties. Excipients and humectants are also added to most hand sanitizers. Medical grade sanitizers usually contain mixture of alcohol and disinfectant (antiseptic) preparations. Most of the commercially used sanitizers are alcohol based with gel, liquid or foam preparations to increase sensory attributes of these preparations [12].

Materials and Methods

We evaluated the in vitro antimicrobial efficacy of ten different hand sanitizers. These included seven commercial (including three herbal) and three of hospital use hand sanitizers. They were anonymized randomly from 1 to 10. The details of their composition and expiry date have been tabulated (Table 1).

Table 1: Showing components of different hand sanitizers (including three herbal and 3 medical grade) along with their date of manufacture and expiry dates.

Sanitizer	Composition	Date of manufacture	Date of expiry
1.	Alcohol IP (Denatured) eq. to Absolute Alcohol 72.34% v/v, water, PEG/PPG-17/6 Copolymer, Propylene Clycol, Acrylates / C10-30 Alkyl Acrylate Crosspolymer, Tetra hydroxypropyl Ethylenediamine, Parfum, Lemonene	12/20	11/22
2.	Ethyl alcohol(denatured) 95% v/v I.P 70%w/w in a perfumed gel base Q.S TO 100%	6/20	12/22
3.	Each 1 ml contains Dhanyaka (*Coriandrum sativum*) Fr. 0.30mg Ushira (*Vetiveriazizanioides*) Rt. 0.30 mg Nagaramusta (*Cyperusscariosua*) Rz. 0.25 mg Hati (*Hedychiumspicatum*) Rz. 0.10 mg Nimba (*Azadirachta indica*) Sd. 0.05mg. Processed in Prasannaq.s (rectified spirit alcohol content 60% v/v)	8/2020	7/2022
4.	Iso-propyl alcohol 70% Acrylate copolymer, triethanolamine, DM water, perfume	06/20	06/23
5.	Active Ingredient - Ethyl Alcohol 70%, Water, Glycerin, PEG-6 (and) AMP - Acrylates/Vinyl Isodecanoate Cross polymer, Diisopropanolamine, Aloe *Barbadensis miller* Leaf Juice.	9/2020	9/2022

6.	Chlorhexidine Gluconate solution 2.5%v/v (Equ. To 0.5% w/v Chlorhexidine Gluconate), Ethyl alcohol IP 70% v/v (with emollient and moisturizer)	6/2019	6/2022
7.	Each 100gm contains 2-propanol IP :45gms 1-propanol :30 gms ethyl-hexadecyl-dimethyl ammonium-ethyl sulphate: 0.2gms. (Mecetronium Ethyl Sulphate)	4/2019	3/2022
8.	Chlorhexidine Gluconate solution 2.5%v/v (Equ. To 0.5% w/v Chlorhexidine Gluconate), Ethyl alcohol IP 70% v/v	Nov 2019	Oct 2021
9.	Alcohol Denatured 73.0%, fresh aloe vera gel 5.0%, Neem Leaf extract 2.0%, Tulsi leaf extract 1.0%,Sweet Orange Essential oil 1.0%, Talisa Patra Leaf extract 0.7%,Vidanga fruit extract 0.5%, Nilgiri leaf extract 0.5%, silver citrate# 0.5%, Coconut water 0.10% Base contains Aqua, Glycerine, Dehydroxanthan Gum, Vitamin E, Phytic acid, Sodium Levulinate & Sodium anisate	04/20	03/22
10.	Ethyl alcohol 70%v/v, aqua, neelam extract, calendula extract, carbome, tea tree oil and aloe vera	June 2021	May 2022

The efficacy of these sanitizers was checked against five ATCC strains: *Staphylococcus aureus* (ATCC 25923), *Staphylococcus hemolyticus* (ATCC 29970), *Enterococcus faecalis* (ATCC 29212), *Pseudomonas aeruginosa* (ATCC 27853) and *Escherichia coli* (ATCC 25922). These bacteria were prepared from lyophilised culture procured from Microbiologics.

This was an in vitro experiment. It was performed in three parts. The first part was performed in triplicates to see the zone of inhibition for each sanitizer[13].The second part was performed to see the duration of action of each hand sanitizer[14] and third part (in triplicate) was performed to see the efficacy of main components (alcohol in different dilutions and disinfectants) separately.

To maintain uniformity of bacterial concentration while culturing, 0.5 McFarland standard was prepared. This solution is known to act as a reference based on which turbidity can be adjusted by varying the concentration of bacteria in the solution. The concentration of bacteria is directly measured by a turbidometry method which is based on the principal of absorbance of solution at a wavelength of 625nm. The reading is proportional to the concentration of bacteria in the solution.

Antimicrobial sensitivity assay

A sterile swab stick was taken and dipped in suspension of respective strains. The extra fluid was squeezed by pressing against the wall of the tube. A lawn /carpet culture of each of the bacterial strain is made on Muller Hinton agar [15].Six wells of 6mm diameter each were punched. Each culture plate had multiple 6mm diameter wells punched using a sterile corkborer. Five of the wells which would be used for sanitizers were made at the periphery and one for control was made in the centre of the plate. As there were ten hand sanitizers to be tested, for each strain of bacteria two culture plates were used. The peripheral wells were filled with 50 microliters of the hand sanitizer using a micropipette. Unaltered concentrations of freshly opened hand sanitizers were used. The centralwell was filled with 50microliters of sterile normal saline as a control. The plates were then incubated for 24 h at 37^{o}C. The zone of inhibition was measured to know the efficacy of different hand sanitizer. The whole experiment was repeated thrice. The experiments were performed thrice for better statistical analysis.

In second part of the experiment we wanted to know the time of action of different sanitizers so 10.0ml of 0.5 McFarland bacterial suspension was prepared. Ten smaller tubes containing 1ml each was prepared from this master mix and were labelled from 1 to 10 for each sanitizer. Further tests were performed sequentially for each sanitizer from 1ml labelled tubes. Gram negative organisms (*Escherichia* coli and *Pseudomonas aeruginosa*) was plated on Blood agar and MacConkey agar each and gram positive (*Staphylococcus aureus, Staphylococcus hemolytics and Enterococcus fecalis*) was plated on blood agar using a loop full (4 mm diameter with 0.01ml of fluid) of the solution. Even if few bacteria were left alive in liquid media they are likely to grow in Blood Agar. Each plate was divided into four parts and streak culture from the above solution was plated at15, 30, 45 and 60 seconds of contact time (of strain and hand sanitizer) respectively. The same procedure was repeated for each sanitizer tube labelled from1 to 10. Each strain was cultured the same way.

As a proof of concept for the third part of experiment individual active components (ethanol, propanol, chlorhexidine and mecetronium ethyl sulphate) of hand sanitizers were used. This included testing multiple dilutions (70%, 80% and 90% respectively) of the both ethanol and propanol along disinfectants 2.5v/v chlorhexidine and 0.2 gmmecetronium ethyl sulphate (in 100ml in distilled water) separately against each of these four bacteria. This experiment was also repeated thrice.

Statistical Analysis

Continuous data is presented asmedian (range) and count data is presented as numbers (proportions). Friedman's non parametric repeated measures ANOVA were used to assess quantitative differences in inhibition zones across multiple test attempts for different hand sanitizers. To avoid the fallacy of multiple hypothesis testing, subgroup differences were only tested if the overall p value was <0.0001. MedCalc Statistical Software version 19.2.6 (Med Calc Software bv, Ostend, Belgium) was used for statistical analysis and all tests were two tailed. Alpha<0.05 was set as significant beforehand. Graphs were populated using Excel and MedCalc.

Statistical Analysis

Staphylococcus hemolyticus

Friedman test

Cases in spreadsheet	3
Cases with missing values	0
Cases included in the analysis	3

Descriptive statistics

	n	Minimum	25th percentile	Median	75th percentile	Maximum
a	3	0.0000	0.000	0.000	0.000	0.000
b	3	0.0000	0.000	0.000	0.000	0.000
c	3	0.0000	0.000	0.000	0.000	0.000
d	3	0.0000	0.000	0.000	0.000	0.000
e	3	0.0000	0.000	0.000	0.000	0.000
f	3	24.0000	24.500	26.000	26.750	27.000
g	3	23.0000	23.500	25.000	25.000	25.000
h	3	25.0000	25.500	27.000	27.750	28.000
i	3	0.0000	0.000	0.000	0.000	0.000
j	3	0.0000	0.000	0.000	0.000	0.000

Friedman test

F	976.0000
DF 1	9
DF 2	18
P	<0.00001

Multiple comparisons

Variable	Mean rank	Different (P<0.05) from variable nr
(1) a	4.0000	(6) (7) (8)
(2) b	4.0000	(6) (7) (8)
(3) c	4.0000	(6) (7) (8)
(4) d	4.0000	(6) (7) (8)
(5) e	4.0000	(6) (7) (8)
(6) f	9.1667	(1) (2) (3) (4) (5) (7) (8) (9) (10)
(7) g	8.0000	(1) (2) (3) (4) (5) (6) (8) (9) (10)
(8) h	9.8333	(1) (2) (3) (4) (5) (6) (7) (9) (10)
(9) i	4.0000	(6) (7) (8)
(10) j	4.0000	(6) (7) (8)

Minimum required difference of mean rank: 0.2334

Staphylococcus aureus

Friedman test

Cases in spreadsheet	3
Cases with missing values	0
Cases included in the analysis	3

Descriptive statistics

	n	Minimum	25th percentile	Median	75th percentile	Maximum
a	3	0.0000	0.000	0.000	0.000	0.000
b	3	0.0000	0.000	0.000	0.000	0.000
c	3	0.0000	0.000	0.000	0.000	0.000
d	3	0.0000	0.000	0.000	0.000	0.000
e	3	0.0000	0.000	0.000	0.000	0.000

	n	Minimum	25th percentile	Median	75th percentile	Maximum
f	3	31.0000	31.250	32.000	33.500	34.000
g	3	19.0000	19.250	20.000	21.500	22.000
h	3	34.0000	34.500	36.000	36.750	37.000
i	3	0.0000	0.000	0.000	0.000	0.000
j	3	0.0000	0.000	0.000	0.000	0.000

Friedman test

Chi-squared	27.0000
DF	9
P	0.0014

Multiple comparisons

Variable	Mean rank	Different (P<0.05) from variable nr
(1) a	4.0000	(6) (7) (8)
(2) b	4.0000	(6) (7) (8)
(3) c	4.0000	(6) (7) (8)
(4) d	4.0000	(6) (7) (8)
(5) e	4.0000	(6) (7) (8)
(6) f	9.0000	(1) (2) (3) (4) (5) (7) (8) (9) (10)
(7) g	8.0000	(1) (2) (3) (4) (5) (6) (8) (9) (10)
(8) h	10.0000	(1) (2) (3) (4) (5) (6) (7) (9) (10)
(9) i	4.0000	(6) (7) (8)
(10) j	4.0000	(6) (7) (8)

Minimum required difference of mean rank: 0.0000

Enterococcus fecalis

Friedman test

Cases in spreadsheet	3
Cases with missing values	0
Cases included in the analysis	3

Descriptive statistics

	n	Minimum	25th percentile	Median	75th percentile	Maximum
a	3	0.0000	0.000	0.000	0.000	0.000
b	3	0.0000	0.000	0.000	0.000	0.000
c	3	0.0000	0.000	0.000	0.000	0.000
d	3	0.0000	0.000	0.000	0.000	0.000
e	3	0.0000	0.000	0.000	0.000	0.000
f	3	22.0000	23.000	26.000	27.500	28.000
g	3	20.0000	20.250	21.000	21.750	22.000
h	3	23.0000	23.250	24.000	25.500	26.000
i	3	0.0000	0.000	0.000	0.000	0.000
j	3	0.0000	0.000	0.000	0.000	0.000

Friedman test

F	324.0000
DF 1	9
DF 2	18
P	<0.00001

Multiple comparisons

Variable	Mean rank	Different (P<0.05) from variable nr
(1) a	4.0000	(6) (7) (8)
(2) b	4.0000	(6) (7) (8)
(3) c	4.0000	(6) (7) (8)
(4) d	4.0000	(6) (7) (8)
(5) e	4.0000	(6) (7) (8)
(6) f	9.5000	(1) (2) (3) (4) (5) (7) (9) (10)
(7) g	8.0000	(1) (2) (3) (4) (5) (6) (8) (9) (10)
(8) h	9.5000	(1) (2) (3) (4) (5) (7) (9) (10)
(9) i	4.0000	(6) (7) (8)
(10) j	4.0000	(6) (7) (8)

Minimum required difference of mean rank: 0.4043

Pseudomonas areuginosa

Friedman test

Cases in spreadsheet	3
Cases with missing values	0
Cases included in the analysis	3

Descriptive statistics

	n	Minimum	25th percentile	Median	75th percentile	Maximum
a	3	0.0000	0.000	0.000	0.000	0.000
b	3	0.0000	0.000	0.000	0.000	0.000
c	3	0.0000	0.000	0.000	0.000	0.000
d	3	0.0000	0.000	0.000	0.000	0.000
e	3	0.0000	0.000	0.000	0.000	0.000

	n	Minimum	25th percentile	Median	75th percentile	Maximum
f	3	23.0000	23.250	24.000	27.750	29.000
g	3	0.0000	0.000	0.000	0.000	0.000
h	3	25.0000	25.250	26.000	27.500	28.000
i	3	0.0000	0.000	0.000	0.000	0.000
j	3	0.0000	0.000	0.000	0.000	0.000

Friedman test

F	180.2500
DF 1	9
DF 2	18
P	<0.00001

Multiple comparisons

Variable	Mean rank	Different (P<0.05) from variable nr
(1) a	4.5000	(6) (8)
(2) b	4.5000	(6) (8)
(3) c	4.5000	(6) (8)
(4) d	4.5000	(6) (8)
(5) e	4.5000	(6) (8)
(6) f	9.3333	(1) (2) (3) (4) (5) (7) (9) (10)
(7) g	4.5000	(6) (8)
(8) h	9.6667	(1) (2) (3) (4) (5) (7) (9) (10)
(9) i	4.5000	(6) (8)
(10) j	4.5000	(6) (8)

Minimum required difference of mean rank: 0.4669

Escherichia coli

Friedman test

Cases in spreadsheet	3
Cases with missing values	0
Cases included in the analysis	3

Descriptive statistics

	n	Minimum	25th percentile	Median	75th percentile	Maximum
a	3	0.0000	0.000	0.000	0.000	0.000
b	3	0.0000	0.000	0.000	0.000	0.000
c	3	0.0000	0.000	0.000	0.000	0.000
d	3	0.0000	0.000	0.000	0.000	0.000
e	3	0.0000	0.000	0.000	0.000	0.000
f	3	22.0000	23.000	26.000	26.750	27.000
g	3	23.0000	23.250	24.000	25.500	26.000
h	3	26.0000	26.000	26.000	26.750	27.000
i	3	0.0000	0.000	0.000	0.000	0.000
j	3	0.0000	0.000	0.000	0.000	0.000

Friedman test

F	136.0000
DF 1	9
DF 2	18
P	<0.00001

Multiple comparisons

Variable	Mean rank	Different (P<0.05) from variable nr
(1) a	4.0000	(6) (7) (8)
(2) b	4.0000	(6) (7) (8)
(3) c	4.0000	(6) (7) (8)
(4) d	4.0000	(6) (7) (8)
(5) e	4.0000	(6) (7) (8)
(6) f	8.8333	(1) (2) (3) (4) (5) (8) (9) (10)
(7) g	8.6667	(1) (2) (3) (4) (5) (8) (9) (10)
(8) h	9.5000	(1) (2) (3) (4) (5) (6) (7) (9) (10)
(9) i	4.0000	(6) (7) (8)
(10) j	4.0000	(6) (7) (8)

Minimum required difference of mean rank: 0.6176

Results:

Box plots of the zone of inhibition of each sanitizer are shown in figure 1with individual data given in supplementary analysis. Sanitizers 1, 2, 3, 4, 5, 9, and 10 failed to inhibit any bacterial growth for all tested organisms. Sanitizers 6 and 8 showed zone of inhibition for all tested gram positive and negative bacteria. Sanitizer 7 showed zone of inhibition for all tested bacteria except *Pseudomonas aeruginosa*.

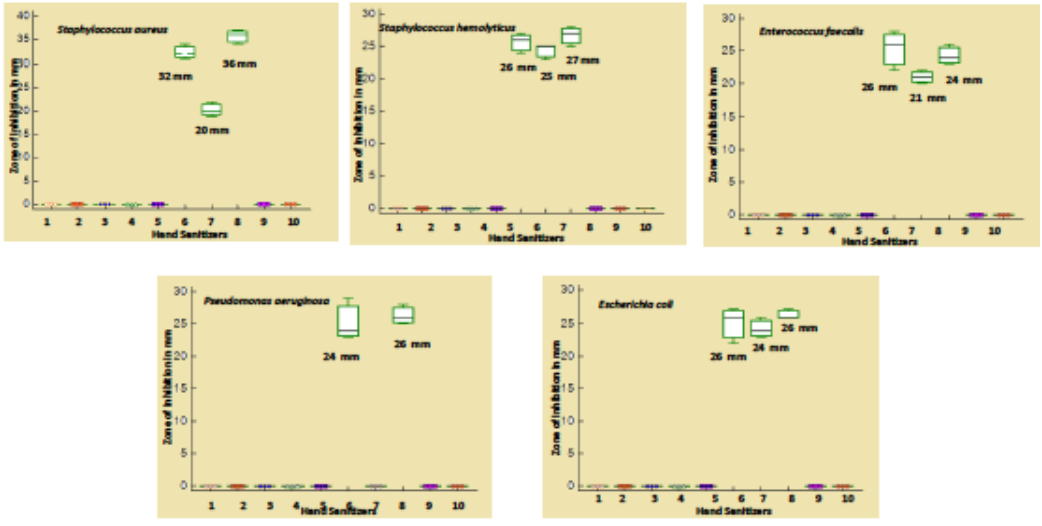


Figure 1: Mean zone of inhibition in mm shown as box plot for Staphylococcus aureus, Staphylococcus hemolyticus, Enterococcus faecalis, Pseudomonas aeruginosa and Escherichia coli.

Results of ANOVA test showing significance of pairwise differences in inhibition zones of different sanitizers for different organisms are shown in supplementary analysis (Table 2).

Table 2: This shows zone of inhibition in mm in triplicate for Staphylococcus aureus, Staphylococcus hemolyticus, Pseudomonas aeruginosa, Escherichia coli, and Enterococcus faecalis

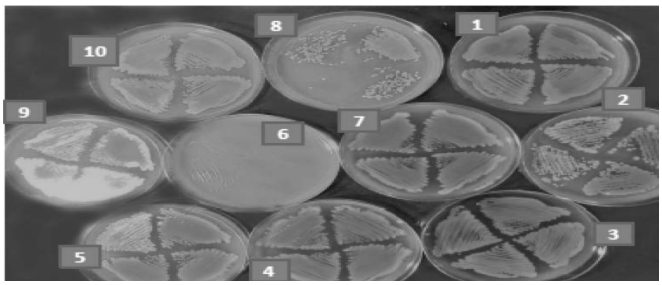
Zone of inhibition in mm Tests in triplicates															
Sanitizer	***Staphylococcus aureus***			***Staphylococcus hemolyticus***			***Enterococcus fecalis***			***Pseudomonas areuginosa***			***Escherichia coli***		
1	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil
2	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil
3	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil
4	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil
5	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil
6	31	34	32	26	27	24	26	28	22	23	29	24	22	27	26
7	22	19	20	25	25	23	22	21	20	Nil	Nil	Nil	24	23	26
8	36	37	34	28	27	25	26	24	23	28	26	25	26	27	26
9	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil
10	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil

To know the effective contact time of sanitizers, each of them was streak cultured after 15, 30, 45 and 60 seconds of contact time. Gram positive bacteria were plated on blood agar alone and gram negative bacteria were plated on blood agar and MacConkey agar each. Results for blood agar showed that for sanitizers 1,2,3,4,5,7,9 and 10 the growth was present at all time points suggesting that no growth was inhibited even after one minute of contact time for any bacteria. Sanitizer 6 inhibited growth for all bacteria at all experimental time points. Sanitizer 8 did not inhibit growth for any bacteria after contact time of 15 seconds. However, it successfully inhibited growth of all bacteria at all subsequent experimental time points (table 3 and figure 2).

Table 3: Table showing of growth for both gram negative and gram positive bacteria on Nutrient Agar after 15, 30, 45 and 60 seconds and on MacConkey agar for gram negative at 15, 30, 45 and 60 seconds (P-growth present, I-growth inhibited).

		Both gram negative and gram positive bacteria on nutrient Agar									
Organism	Time in seconds	Sanitiser 1	Sanitiser 2	Sanitiser 3	Sanitiser 4	Sanitiser 5	Sanitiser 6	Sanitiser 7	Sanitiser 8	Sanitiser 9	Sanitiser 10
E.coli	15	P	P	P	P	P	I	P	P	P	P
	30	P	P	P	P	P	I	P	I	P	P
	45	P	P	P	P	P	I	P	I	P	P
	60	P	P	P	P	P	I	P	I	P	P
Pseudomonas	15	P	P	P	P	P	I	P	P	P	P
	30	P	P	P	P	P	I	P	I	P	P
	45	P	P	P	P	P	I	P	I	P	P
	60	P	P	P	P	P	I	P	I	P	P
S.aureus	15	P	P	P	P	P	I	P	P	P	P
	30	P	P	P	P	P	I	P	I	P	P
	45	P	P	P	P	P	I	P	I	P	P
	60	P	P	P	P	P	I	P	I	P	P
Enterococcus	15	P	P	P	P	P	I	P	P	P	P
	30	P	P	P	P	P	I	P	I	P	P
	45	P	P	P	P	P	I	P	I	P	P
	60	P	P	P	P	P	I	P	I	P	P
S.hemolytics	15	P	P	P	P	P	I	P	P	P	P
	30	P	P	P	P	P	I	P	I	P	P
	45	P	P	P	P	P	I	P	I	P	P
	60	P	P	P	P	P	I	P	I	P	P
				Gram negative bacteria on McConkey Agar							
Organism	Time in seconds	Sanitiser 1	Sanitiser 2	Sanitiser 3	Sanitiser 4	Sanitiser 5	Sanitiser 6	Sanitiser 7	Sanitiser 8	Sanitiser 9	Sanitiser 10
E.coli	15	P	P	P	P	P	I	P	P	P	P
	30	P	P	P	P	P	I	P	I	P	P
	45	P	P	P	P	P	I	P	I	P	P
	60	P	P	P	P	P	I	P	I	P	P
Pseudomonas	15	P	P	P	P	P	I	P	P	P	P
	30	P	P	P	P	P	I	P	I	P	P
	45	P	P	P	P	P	I	P	I	P	P
	60	P	P	P	P	P	I	P	I	P	P

Figure 2: Image showing growth patterns all ten hand sanitizers on MacConkey agar for *Escherichia coli*



For the third part of experiment, growth was seen at all dilutions of ethanol and propanol when tested against five ATCC bacteria (Table 4).However, disinfectant 2.5v/v chlorhexidine was able to inhibit all fivebacteria.0.2 gm mecetronium ethyl sulphate showed inhibition for *Staphylococcus aureus*, *Staphylococcus hemolyticus*, *Enterococcus faecalis* and*Escherichia coli*. However, *Pseudomonas aeruginosa* was not inhibited by this disinfectant.

Table 4: Dilutions of various disinfectant agents- ethyl alcohol and propanol, chlorhexidine, mecetronium ethyl sulphate tested against *S. hemolyticus, S. aureus, Enterococcus, Escherichia coli* and *Pseudomonas aeruginosa* (in triplicates).

Dilutions of various disinfectant agents tested against *S. hemolyticus*, Zone of inhibition in mm±1mm																							
	Ethanol									Propanol									Chlorhexidine			Mecetronium ethyl sulphate		
S.N	*60%*			*70%*			*80%*			*60%*			*70%*			*80%*			*2.5 v/v*			*0.2 gm in 100ml*		
1	*Nil*	*Nil*	*Nil*	*Nil*	*Nil*	*Nil*	*Nil*	*Nil*	*Nil*	*Nil*	*Nil*	*Nil*	*Nil*	*Nil*	*Nil*	*Nil*	*Nil*	*Nil*	*32*	*33*	*36*	*15*	*12*	*15*
Dilutions of various disinfectant agents tested against *S. aureus*																								
	Ethanol									Propanol									Chlorhexidine			Mecetronium ethyl sulphate		
S.N	60%			70%			80%			60%			70%			80%			2.5 v/v			0.2 gm in 100ml		
2	*Nil*	*Nil*	*Nil*	*Nil*	*Nil*	*Nil*	*Nil*	*Nil*	*Nil*	*Nil*	*Nil*	*Nil*	*Nil*	*Nil*	*Nil*	*Nil*	*Nil*	*Nil*	36	32	39	12	17	19
Dilutions of various disinfectant agents tested against *Enterococcus*																								
	Ethanol									Propanol									Chlorhexidine			Mecetronium ethyl sulphate		
S.N	60%			70%			80%			60%			70%			80%			2.5 v/v			0.2 gm in 100ml		
3	*Nil*	*Nil*	*Nil*	*Nil*	*Nil*	*Nil*	*Nil*	*Nil*	*Nil*	*Nil*	*Nil*	*Nil*	*Nil*	*Nil*	*Nil*	*Nil*	*Nil*	*Nil*	26	26	29	22	17	22
Dilutions of various disinfectant agents tested against *Escherchia coli*																								
	Ethanol									Propanol									Chlorhexidine			Mecetronium ethyl sulphate		
S.N	60%			70%			80%			60%			70%			80%			2.5 v/v			0.2 gm in 100ml		
4	*Nil*	*Nil*	*Nil*	*Nil*	*Nil*	*Nil*	*Nil*	*Nil*	*Nil*	*Nil*	*Nil*	*Nil*	*Nil*	*Nil*	*Nil*	*Nil*	*Nil*	*Nil*	27	26	34	26	19	28
Dilutions of various disinfectant agents tested against *Pseudomonas*																								
	Ethanol									Propanol									Chlorhexidine			Mecetronium ethyl sulphate		
S.N	*60%*			70%			80%			60%			70%			80%			2.5 v/v			0.2 gm in 100ml		
5	*Nil*	*Nil*	*Nil*	*Nil*	*Nil*	*Nil*	*Nil*	*Nil*	*Nil*	*Nil*	*Nil*	*Nil*	*Nil*	*Nil*	*Nil*	*Nil*	*Nil*	*Nil*	24	27	26	*Nil*	*Nil*	*Nil*

Discussion

Our *in vitro* study had three parts. The first part showed that only sanitizers with two active antimicrobial ingredients worked, second part showed that hand sanitizer containing ethanol and chlorhexidine was fastest to act and third part showed that when tested individually in serial dilutions, chlorhexidine was the most effective followed by mecetronium ethyl sulphate. These findings clearly showed that medical grade hand sanitizers (number6, 7 and 8) performed better than commercial hand sanitizers. Medical grade hand sanitizers have disinfectants added to them which make them more effective by prolonging the effect of alcohol based hand sanitizers. This has also been demonstrated by Alyet al and Kampf(1979), G et al (1999) in their work [16,17,18].

Ourstudy is an in vitro study and hand sanitizers containing only alcohol as their main ingredient were unable to inhibit growth of any of the bacteria. Out of the three, two medical grade hand sanitizers which had ethyl alcohol and chlorhexidine gluconate as their main ingredient (6 and 8) performed better than the third (7) hand sanitizer which had propanol and mecetronium ethyl sulphate as their main ingredient.

The second part of our experiment was performed to know time of action of different sanitizers. Culture plates were streak cultured at 15, 30, 45 and 60 seconds of contact time and inhibition of growth was noted. Here hand sanitizer 6 (growth inhibition started after 15 seconds) performed the best in blood agar and MacConkey for both gram positive and gram negative bacteria. Hand sanitizer 8 was able to inhibit growth in both medium and for all bacteria after 30 seconds. Besides this, other hand sanitizers including 7 (which had shown growth inhibition in earlier experiment for *Staphylococcus aureus* and *Staphylococcus hemolyticus)* were unable to show any growth inhibition up to 60 seconds. WHO based formulations are known to be have bactericidal activity within 60 seconds [19]. Our in vitro experiments were able to show adequate activity of hand sanitizers within 60 seconds for only three sanitizers.

Third part of the experiment also showed that individual dilutions of alcohol were also not able to inhibit both gram positive and gram negative growth. This confirmed that both ethanol and propanol were ineffective at 60%, 70%, 80% (v/v) concentrations against all five bacteria. Disinfectants individually especially 2.5 v/v chlorhexidine performed the best followed by 0.2 gm mecetronium ethyl sulphate in 100ml distilled water which was unable to inhibit *Pseudomonas aeruginosa*. Suchomel et al in their review article published in 2020 have highlighted that most of hand sanitizer are based on 2009 WHO formulations have alcohol in volume/volume (v/v) percentages which are not having sufficient activity against the bacteria. They have suggested modifications by increasing the amount of alcohol by ~7% which is done by using weight (w/w) instead of volume percent concentrations. They have also suggested decreasing the glycerol from 1.45% to 0.725% in WHO preparations [20]. European Committee for Standardization European Norm (EN) 1500 has also suggested higher concentrations of alcohol in weight/weight (w/w) for adequate efficacy of hand rubs [21].

Our study had few limitations. It is an in vitro experiment with artificially created very high load of bacteria. These findings however set a ground for further studies to perform in vivo experiments and validate the claims set forth in these experiments.

Conclusion

To conclude this is an in vitro study which highlights that hand sanitizers with both alcohol and disinfectants as their main ingredient perform better than alcohol only based hand sanitizers. This also preludes for further in vivo studies with higher concentrations (w/w) of alcohol to validate modified WHO hand sanitizer preparations.

Funding Source: This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

Acknowledgement: None

Competing interests: None

References

1. Grice, E.A.; Segre, J.A., 2011,“The skin microbiome”, Nat. Rev. Microbiol., 9, pp. 244–253.
2. Carter S J 2000, “Aseptic technique Cooper and Gunn’s Dispensing for Pharmaceutical Students”, 12th Edition, CBS Publishers and Distributors, pp. 494-540.
3. Hübner NO, Hübner C, Wodny M, Kampf G, Kramer A., 2010,“Effectiveness of alcohol-based hand disinfectants in a public administration: impact on health and work performance related to acute respiratory symptoms and diarrhoea”, BMC Infect Dis., 10:250. doi: 10.1186/1471-2334-10-250.
4. Tambekar DH, Shirsat SD, Suradkar SB, Rajankar PN, Banginwar YS, 2007,” Prevention of transmission of infectious disease: Studies on hand hygiene in health-care among students”, Cont J Biomed Sci. ,1,pp 6–10.
5. Larson EL, Hughes CA, Pyrek JD, Sparks SM, Cagatay EU, Bartkus JM., 1998, “Changes in bacterial flora associated with skin damage on hands of health care personnel”. Am J Infect Control., 26, pp.513–21.
6. Nuland SB,” The doctor’s plague: germs, childbed fever, and the strange story of Ignác Semmelweis”, New York: WW Norton & Company, 2003.
7. Centers for Disease Control and Prevention 2002.Guideline for hand hygiene in health-care settings. Recommendation of the Healthcare Infection Control Practices Advisory Committee and the HICPAC/SHEA/APIC/IDSA Hand Hygiene Task Force.
8. Pickering AJ, Boehm AB, Mwanjali M, Davis J, 2010. ”Efficacy of waterless hand hygiene compared with handwashing with soap: a field study in Dar es Salaam, Tanzania”, Am J Trop Med Hyg. 82, 2, pp.270-8. doi:10.4269/ajtmh.2010.09-0220.
9. Pittet D, Allegranzi B, Boyce J., 2009. ”World Health Organization World Alliance for Patient Safety First Global Patient Safety Challenge Core Group of Experts. The World Health Organization Guidelines on Hand Hygiene in Health Care and their consensus recommendations. “,Infect Control Hosp Epidemiol, 30,7, pp. 611-22. doi: 10.1086/600379.

10. Jing JLJ, Pei Yi T, Bose RJC, McCarthy JR, Tharmalingam N, Madheswaran T., 2020. ”Hand Sanitizers: A Review on Formulation Aspects, Adverse Effects, and Regulations. Int J Environ Res Public Health.” , 11,17,9,pp. 3326. doi: 10.3390/ijerph17093326.

11. Gold, N.A.; Avva, U 2018. Alcohol Sanitizer; StatPearls Publishing: St. Petersburg, FL, USA.

12. Greenaway, R.E.; Ormandy, K.; Fellows, C.; Hollowood, T., 2018,” Impact of hand sanitizer format (gel/foam/liquid) and dose amount on its sensory properties and acceptability for improving hand hygiene compliance.”, J. Hosp. Infect., 100, pp. 195–201.

13. Jain VM, Karibasappa GN, Dodamani AS, Prashanth VK, Mali GV. , 2016 ,“Comparative assessment of antimicrobial efficacy of different hand sanitizers: An in vitro study.”Dent Res J (Isfahan)., 13,5, pp. 424-431. doi:10.4103/1735-3327.192283

14. Payne DN, Babb JR, Bradley CR., 1999,” An evaluation of the suitability of the European suspension test to reflect in vitro activity of antiseptics against clinically significant organisms.”Lett Appl Microbiol., 28,1, pp.7-12. doi:10.1046/j.1365-2672.1999.00482.x

15. Paniker, J., 2005, Ananthanarayan and Paniker's textbook of microbiology, Himayatnagar, Hyderabad : Orient Longman

16. Aly R, Maibach HI., 1979,“Comparative study on the antimicrobial effect of 0.5% chlorhexidine gluconate and 70% isopropyl alcohol on the normal flora of hands. Appl Environ Microbial.”,37,3, pp. 610-3. doi: 10.1128/aem.37.3.610-613.1979.

17. Kampf G, Höfer M, Wendt C., 1999. ”Efficacy of hand disinfectants against vancomycin-resistant enterococci in vitro. “J Hosp Infect., 42,2, pp. 143-50. doi: 10.1053/jhin.1998.0559.

18. Jing JLJ, Pei Yi T, Bose RJC.2020. “Tharmalingam N, Madheswaran T. Hand Sanitizers: A Review on Formulation Aspects, Adverse Effects, and Regulations. Int J Environ Res Public Health. 11;17(9):3326. doi: 10.3390/ijerph17093326. PMID: 32403261; PMCID: PMC7246736.

19. Suchomel M, Kundi M, Pittet D, Weinlich M, Rotter ML.,2012, ”Testing of the World Health Organization recommended formulations in their application as hygienic hand rubs and proposals for increased efficacy. “,Am J Infect Control., 40,4, pp. 328-31. doi: 10.1016/j.ajic.2011.06.012.

20. Suchomel M, Steinmann J, Kampf G.,2020,” Efficacies of the original and modified World Health Organization-recommended hand-rub formulations.” J Hosp Infect., 106,2, pp. 264-270. doi: 10.1016/j.jhin.2020.08.006. PMID: 32800826; PMCID: PMC7423627.

21. European Committee for Standardization. European Norm (EN) 1500. Chemical disinfectants and antiseptics—hygienic hand disinfection—test method and requirement (phase 2/ step 2). Brussels: The Committee; 2018.

22. Suchomel M, Eggers M, Maier S, Kramer A, Dancer SJ, Pittet D, 2020,”Evaluation of World Health Organization-Recommended Hand Hygiene Formulations.”, Emerg Infect Dis. ,26,9, pp. 2064-2068. doi: 10.3201/eid2609.201761.