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EFFECT ON POLY-MICROBIAL SURVIVAL UNDER DYNAMIC CONDITION TO DETERMINE SUPERIORITY OF HAND WASH FORMULATION

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Abstract

A novel method to co-relate the efficacy of handwash formulations to real situation was developed and tested. We have used static and dynamic state of microbes to evaluate the efficacy. The findings clearly show that the viscosity of the formulation, its solubility, the percentage of insoluble constituents, spreadbility etc., plays greater role in the removal of germs than any other aspect. Further the rate of removal of microbes from different regions of the palm such as thumb, index, middle, ring, little finger tips as indicator of the performance of the formulation. Details are presented in the paper.

Introduction

The importance of hand hygiene in preventing various microbial diseases got a new lease of life with the general public all over the world with the advent of novel coronavirus (SARS- COV-2) and COVID19. [1, 2, 3]

Palmar region, fingertips and nailbed are known to harbor wide variety of microbes including the pathogens and the pathogens from such source are likely to auto-inoculate as well as transmits on to others directly or via several extra-human reservoirs such as door knob, utensils, direct contact etc. Therefore hand asepsis is necessary to control the pathogen transmission, disease outbreak and the subsequent extension of the disease to become epidemic if the pathogen is highly contagious. [4, 5, 6]

The hand sanitizers (alcohol based) and hand wash formulations are conventionally made with antiseptics and anti-microbials with the assumption of eliminating the microbes from the palmar region.[7,8] However the contact times of the sanitizer(s) is very important to kill the microbes and if the contact time is short due to the volatile nature of the formulation, the effect will be poor.

In the case of hand wash formulations, several factors such as viscosity, pH, complete miscibility in water, the amount of insoluble constituents in the formulation and quick release of the antimicrobials from the formulation are known to influence the efficacy.

The microbes in the palmar region would be in a dynamic state due to the diverse species of microbes present in any given unit area in the palm.[9, 10] Therefore the conventional anti-microbial assay may not help to predict the efficacy of handwash preparations against wide spectrum of microbes due to high 'dynamicity', diversity of microbes in the palmar region.

We have devised a novel method to evaluate the anti-microbial activity of 3 handwash formulations wherein the method of evalution of the efficacy was devised in such a way to directly interferes at the dynamic level of growth of the organism.

The efficacy of the anti-microbials against the microbes during dynamic culture condition was co-related directly with reduction in microbial abundance in the palmar region.

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Our novel method may turns to become a new saga in studying the anti-microbials to achieve precise targeting method for testing anti-microbials. We have included both kinetic and kinematics of microbiome in the palmar region for the study. Details are presented in the paper

Materials and methods

Enumeration of microbial abundance in the palmar region (before treatment)

Five volunteers actively involved in diverse activity at shop-floor and likely to gather and transmit several microbes were chosen for the study. Hand impression was made over a petri dish containing nutrient agar in quadruplet. The total number of different species of microbe and the number of individual microbes were calculated and then the relative abundance of key indicator species was calculated. For the present study we have included only Staphylococcus aureus, Pseudomonas, Klebsiella and corynebacterium as indicator species.

All the above organism were isolated and grown separately and were identified by standard microbiological procedure.

Anti-microbial assay by spread plate method [11, 12]

Different concentrations of the 3 handwash formulations were weighed and incorporated with 15ml of both nutrient agar, homogenized with the media and then allowed to solidify. The organism with definite inoculum size was poured over the surface of the media. After 24 and 48hrs of incubation the plates were observed and the growth and number of colonies were tabulated. The concentrations of handwash formulation used in the assay were 1, 3 and 5% respectively. The percentage of reduction vis-à-vis concentration was calculated.

Anti-microbial assay -Zone of inhibition

In a nutrient agar plate the organism was swabbed over the media to achieve even and uniform growth of the organism. After 10 minutes of inoculation a well of 1cm diameter was bored and then 200µl of 1, 3 and 5% solution of 3 handwash preparations were loaded separately into the well, the plates were incubated for 24hrs and the zone of inhibition was measured.

Antimicrobial assay under dynamic culture

100ml of nutrient broth was prepared in a sterile conical flask and 100 $x10^2$ inoculum of each species of microbe was inoculated and then the broth was treated with 1, 3 and 5% of different handwash formulation. The erylenmeyer flask were kept in rotary shaker for 12 and 24 hours and the OD was taken at 280 nm and from the control value the confluence of microbial growth and percentage reduction were calculated.

Invivo microbial reduction assay in human volunteers

The 3 different handwash formulations were prepared in water in the following concentrations such as 1, 3, and 5% respectively. The palmar regions including the nailbed were washed with the above formulations and the washing time (contact time) was maintained as 2 minutes. After 2 minutes the hand was washed with copious amount of water and then the palmar region was gently pressed over the surface of nutrient agar plate. The number of colonies of different species of organism grown vis-à-vis the region of palm were counted and the efficacy of handwash was interpreted.

Quantity of water required for the complete removal of hand wash formulations

An experiment was conducted to evaluate the quantity of water required to remove the handwash completely between the test sample versus two market samples. The feel of the user was taken as the endpoint.

Percentage of insoluble constituents in the 3 formulation

10gm of each sample was weighed and dissolve in 100ml of water in a conical flask and the entire setup was undisturbed for an hour. The amount of insoluble matter in each handwash formulations settled at the bottom was weighed and percentage insoluble matter was calculated.

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Results

Enumeration of microbial abundance in the palmar region (Data shown in the table is average of 5 volunteers, irrespective of size, numbers only taken)

In the present study we have taken only 4 species of microbes and excluded other 11 species of microbe isolated. For calculating relative abundance the total number of colonies of 4 species alone was used. Staphylococcus was the most abundant species of microbe with 80%, followed by Pseudomonas. Table- 1

Species	No. of colonies	Relative abundance in %
Staphylococcus	102	80
Pseudomonas	18	14
Klebsiella	6	5
Corynebacterium	2	2
Total	128	100

Anti-microbial assay by spread plate method

The handwash formulation 1 showed highest activity with 80 % and above inhibition of colony forming units of all the key indicator species. Whereas the 2 other hand wash formulation such as 2 & 3 did not show great anti-microbial activity. Table- 2

Organism	% reduction in CFU								
	Handwash 1 (%)			Handwash 2 (%)			Handwash 3 (%)		
	1	2	3	1	2	3	1	2	3
Staphylococcus aureus	**40**	**60**	**80**	**20**	**30**	**40**	**18**	**25**	**38**
Pseudomonas	**64**	**78**	**90**	**30**	**34**	**40**	**40**	**45**	**52**
Klebsiella	**52**	**64**	**80**	**20**	**28**	**32**	**34**	**42**	**62**
Corynebacterium	**62**	**79**	**92**	**30**	**44**	**52**	**60**	**70**	**82**

Anti-microbial assay – Zone of inhibition

The handwash formulation 1 showed great anti-microbial activity when compared to other two formulations. Table-3

Organism	Zone of inhibition in mm								
	Handwash 1 (%)			Handwash 2 (%)			Handwash 3 (%)		
	1	2	3	1	2	3	1	2	3
Staphylococcus aureus	20	29	34	11	13	13	8	11	14
Pseudomonas	24	26	29	12	12	14	10	15	18
Klebsiella	28	34	38	8	12	15	11	18	21
Corynebacterium	32	38	41	11	8	18	14	20	25

Antimicrobial assay under dynamic culture

The handwash formulation 1 exhibited very high microbe growth limiting effect under dynamic condition against all the 4 microbes studied. Irrespective of the concentration the activity was more or less the same, whereas, the other 2 handwash formulations showed inferior activity against all the 4 microbes studied. Table- 4

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Organism	% reduction based on OD value								
	Handwash 1 (Conc. %)			Handwash 2 (Conc. %)			Handwash 3 (Conc. %)		
	1	2	3	1	2	3	1	2	3
Staphylococcus aureus	60	72	80	32	43	45	28	29	32
Pseudomonas	40	48	52	25	39	42	22	31	35
Klebsiella	44	52	59	29	31	48	18	21	28
Corynebacterium	60	78	85	27	35	39	25	31	41

Invivo microbial reduction assay in human volunteers

Handwash formulation 1 has reduced the microbial abundance in the palm (different regions) by 99.9% as against 80 & 87% reduction of microbes respectively for the formulations 2 &3. Table- 5

Test	Quantity of product used	Microbial load in CFU/2cm^2 area					
		Thumb	Index	Middle	Ring	Little	Overall reduction
Before	-	>50,000	>60,000	>100,000	>80,000	>70,000	-
Handwash-1	1ml	>30,000	>20,000	>30,000	>20,000	>20,000	66.6
	2ml	>8,000	>5,000	>12000	>7000	>6000	89.4
	3 ml	>100	>100	>100	>100	>100	99.8
	5ml	>50	>50	>50	>50	>50	99.9
Handwash-2	1ml	>48,000	>42,000	>60,000	>30,000	>30,000	41
	2 ml	>20,000	>15,000	>32000	>18000	>16000	71
	3 ml	>17,000	>11,000	>15,000	>14,000	>13,000	80
	5ml	>150	>100	>100	>100	>100	99.8
Handwash-3	1ml	>32,000	>21,000	>40,000	>24,000	>19,000	62
	2 ml	>11,000	>9,000	>15,000	>12,000	>11,000	83
	3 ml	>9,000	>10,000	>7,000	>11,000	>8,000	87
	5ml	>90	>100	>100	>100	>100	99.8

Quantity of water required for the complete removal of hand wash formulations

100 ml of water was sufficient to remove handwash formulation 1, whereas 400ml of water was required for the removal of handwash formulation 2 &3. Table- 6

Formulation	Quantity of water used in ml			
	100	200	300	400
Handwash- 1	NR	NR	NR	NR
Handwash- 2	RP	RP	RP	NR
Handwash- 3	RP	RP	RP	NR

NR- No residue; RP- Residue present

Percentage of insoluble constituents in the 3 formulation

The percentage of insoluble constituents was evaluated to understand the likely easy permeation of handwash formulation during use. The handwash formulation had hardly any water insoluble constituents whereas the formulation 2 & 3 showed water insoluble matter more than 10%. Table- 7

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Formulation	% insoluble water
Handwash- 1	< 0.5
Handwash- 2	12
Handwash- 3	11

Discussion

We are the first to attempt to study the efficacy of handwash formulations against several key indicator microbes in the palmar region under dynamic conditions.

The conventional anti-microbial assays whether it is pour plate or spread plate or zone of inhibition, all assays are done against the microbes in a static condition. In the static condition the test compound is incorporated in the media and the organism is then grown in the media. Under such situation, if the organism could able to move to a latent phase for a transient while the organism can comes back to active phase once the test compound gets neutralized in the media.

The water immiscibility of the compounds in the formulation also may affect the antimicrobial activity. Whereas, in dynamic condition, the organism is continuously supplied with oxygen due to the constant movement of the culture condition offers a regulated provisioning of the essential nutrients. Therefore the organism has enough space to overpower the anti-microbial activity if possible.

It is well established that the microbial growth will be several fold higher in shake culture than in stationary culture. Therefore the arrest of growth of any microorganism under shake culture otherwise called as dynamic condition would mean a lot about the 'true' antimicrobial activity of test compound.

The handwash formulation1 showed remarkable antimicrobial activity against all the four indicator microbes tested. Further, both the conventional assays such as spread plate and zone of inhibition also yielded superior antimicrobial activity for the formulation1 when compared to formulation 2& 3.

The amount of water insoluble constituents in formulation1 was less than 0.5% whereas other two formulations had about 10%. The water miscibility, viscosity and the amount of water insoluble constituents may be limiting the performance of the formulations such as 2 & 3 whereas the formulation 1 was completely miscible in water. The viscosity of formulation1 was 3 fold lower than 2 & 3 and had also only below 0.5% water insoluble constituents.

The constrains inbuilt in the formulation of 2 & 3 will certainly make the formulations not so easily miscible in water and further 10% of water insoluble constituents are likely to trap and immobilize the antimicrobial present and thereby would affect the performance.

The formulation1 exhibited perfect correlation between in vitro and in vivo results. Further the quantity of water required to remove the residual effect of formulation1 was 100ml whereas formulation 2 & 3 demanded 400ml of water. When greater the quantity of water required to remove the handwash means the proportion of water required also would be higher for its dispersal.

The formulation1 is composed of herbal and chemical complex (extracts of Curcuma, Ocimum and Azadirachta along with cetrimide, benzalkonium chloride and thymol). The formulation 2 & 3 are composed of chemicals which are mere surfactants and have broad non-specific antimicrobial activity.

The formulation intelligence and the careful selection of actives based on the evaluation performed under dynamic condition is the reason for the formulation1 to be superior over formulation 2&3.

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Our study findings clearly indicate that less viscous, highly water miscible handwash formulations may exhibit superior activity than highly viscous handwash preparations. The less viscous, highly water miscible formulations can disperse easily in water and can reach each and every grain in the palmar region, phalanges, nailbed quickly and can also remove the pathogen during handwash.

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