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Research Article

A Novel Foot Bath for Horse Dwelling

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ABSTRACT

Ensuring you have good biosecurity measures in horse dwelling will ensure your horse stays safe and well. An important component of any biosecurity programs is controlling the mechanical transmission of pathogens by staff members via disinfectant foot bath outside stables, In our work, we studied the effect of five disinfectants used as foot bath in three models (liquid and semiliquid and dry) to evaluate their effects against *streptococcus equi sub species equi* isolated from native horse breed in Egypt, the tested strain of *S. equi subsp. equi* showed antibiotic resistance against tetracycline, cortimoxazole, rifampicin and cephalothin, the results of samples taken from the foot bath for three successive days showed, increasing the count of strept.equi day after day in the three model types especially dry, regarding strept equi count log reduction of rubber coupons after one minute contact in different types of foot bath for three successive days, the semiliquid foot bath showed the best results as the reduction was improved for all types of disinfectants used that showed bacterial log reduction >5 except formalin (4.04) and staldren (3.35), followed by liquid model followed by the dry which showed the worst results, in conclusion, The footbath as a simple form of biosecurity is usually soiled with microorganism and should be frequently replaced to avoid to be useless or to be a source of infection specially in the liquid form, semiliquid foot bath prolonged the action of disinfectant when used and improve bactericidal activity when compared with liquid and dry form.

Key words: Footbath, *S. equi subsp. equi*, Disnfectants and Antibiotic resistance

INTRODUCTION

Biosecurity in horse farms means prevention of infectious diseases in horses involves two main tactics: vaccination and disinfection. Although many adequate vaccines are commercially available, none can be guaranteed to be 100% effective. Even with proper vaccination. For several disease-causing organisms which can cause major outbreaks of disease, no vaccine is currently available. This is also the case for the bacteria which cause septicemia in newborn foals. Given that vaccination is not universally effective for disease prevention, disinfection management practices are essential in providing a healthy environment for horses. Adequate discussion of the disinfection of equine premises requires examination of the pathogens encountered the surfaces to be disinfected. Biosecurity is a group of preventive measures considered to decrease the risk of spread of infectious diseases (Koblentz, 2010). Shoe soles are possible vectors for infectious diseases. Although studies have been occurred to measure the prevalence of infectious pathogens on shoe soles and decontamination procedures, no systematic study has ever occurred. The footbath is a very simple form of biosecurity that helps prevent the potential spread of disease. Organisms have the potential to survive for several days or weeks in the dirt stuck to the bottom of your shoes. Footbaths can remove these organisms. Foot mats and footbaths can serve as visual indicators to personnel that they are entering or leaving areas of greater risk within a facility and the Reductions in colony-forming units (CFUs) on treated boots were varied by disinfectant (Hornig *et al*., 2016) on the other hand, Stockton *et al.*, 2006 concluded that, the numbers of aerobic bacteria recovered from floor surfaces were not affected by use of rubber over boots or the types of disinfectant used in the study. Studies have shown that disinfectant foot mats have variable efficacy for decreasing bacterial contamination of footwear or flooring and that the type of disinfectant can be critical to obtaining maximal reductions in bacterial counts (Dunowska *et al*. 2006). *Streptococcus equi* is an important equine pathogen classified under Lancefield group C *streptococci* with 2 subspecies of major clinical

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relevance in horses: *S. equi subsp. equi* and *S. equi subsp. zooepidemicus*. S. *equi subsp. equi* is the etiologic agent of strangles, a highly contagious and severe nasopharyngeal disease of horses (Mallicote, 2015; Rinosh *et al*., 2017). The disease is transmitted through inhalation or direct contact with mucopurulent discharge from an infected animal, resulting in fever, depression, and enlargement of the submandibular and retropharyngeal lymph node that can lead to respiratory distress. Complications include secondary cellulitis at external abscessation sites, guttural pouch empyema and its persistence into the carrier state, purpura haemorrhagica, metastatic abscessation, emergency tracheostomies and seldom secondary *S. equi* pneumonia or myositis. (Boyle, 2017). A person handling an infected horse can carry the organism on clothing, boots, or unwashed hands. One of the most important challenges facing equine practitioners is to effectively Control which help to prevent highly contagious Streptococcus equi sub species equi infections or strangles.

Resistance to antibiotics has been increased which knocked the door of worry and restless which will give alarm of difficult treatment and also antibiotic resistance genes transmission so we aimed to detect antibiotic resistance of *S. equi subsp. equi* and examined it against some disinfectants targeting to control spread of infection and decrease usage of antibiotics. Control of outbreaks requires strict isolation protocols and hygiene measures Amass and Clarck, (1999) stated that boot baths are usually poorly maintained on farms and are frequently soiled with organic matter. Disinfectant footbaths should not be expected to sterilize footwear, but they may help in reducing the risk for nosocomial infection when used with effective disinfectants (Morley *et al*., 2005).

Our study aimed to evaluate Three foot bath model systems (liquid, semi liquid and dry model) performed at the lab using five disinfectants (Calcium hypoclorite 5%, Halamid 5%, Staldren 5%, virkon S 2% and formalin 5% or paraformaldehyde powder 5% conc.) and evaluated against the *strept equi sub species equi* stain isolated from native horse breed farms in Egypt.

MATERIALS AND METHODS

Tested organism

A local strain of *S. equi subsp. equi* obtained from native breed was cultivated on Sheep blood agar medium (Oxoid)according to Quinn *et al.*, 2011 and identified by PCR using specific primer to *tuf* gene specific to the *Streptococcus sp*. and primer specific to eqbe gene specific to *S. equi subsp. equi* according to Picard *et al*., 2004 and North *et al.*, 2014 then tested against different antimicrobial agents to examine its antibiotic resistance by disk diffusion test against the following antibacterial discs according to the Clinical and Laboratory Standards Institute (CLSI, 2015).

Preparation of bacterial suspension for foot bath evaluation

Cultures of streptococcus *strept equi sub species equi* was inoculated into 9 ml of Buffered peptone water (BPW). Reconstituted cultures were incubated at 37°C for 24 h. One ml of the overnight culture was inoculated into a test tube containing 9 ml tryptic soy broth, diluted 1:10 (Tryptic soy broth TSB).

Disinfectant

Neutralizing agent

Neutralizing agent was prepared according to (Horejsha and Kampf 2010): For all disinfectants {3% Tween 80 (polysorbate 80) (Mp Biomedicalis), 0.3 Lethcine (Fisher chemicals), 1% Histidine (Fisher chemicals), 0.5% Sodium thiosulphate (Fisher chemicals), 3% Saponine (Fisher chemicals)}.

Coupons

Rubber coupons (3cmx3cm in diameters) of same materials as that used in the sole of the shoes of the stockmen in farms, the coupons were sterilized by autoclaving at 121 for 15 minutes before use.

Antibiogram assay

Susceptibility of the *S. equi subsp. equi* was determined against 8 antimicrobial agents commonly used for treatment and considered as important agents for antimicrobials used, included in this study were erythromycin (15 µg), tetracycline (30 µg), clindamycin (2 µg), Cotrimoxazole (25 µg), rifampicin (5 µg), chloramphenicol (30 µg), Cephalothin (30 µg), and vancomycin (30 µg). The strain was inoculated into Mueller–Hinton broth (Oxoid) and incubated overnight at 37C. The turbidity of the suspensions was adjusted to a 0.5 McFarland standard and streaked onto Mueller–Hinton agar (Oxoid) plates. Antimicrobial disks were added on the plates and they were incubated aerobically at 37ºC for 16–18 h. The results were recorded as susceptible, intermediate or resistant by measurement of the inhibition zone diameter according to the zone diameter interpretative standards of CLSI (2015) or according to the antimicrobials manufacturer's instructions.

Foot baths

Three foot baths model systems were used, liquid, semi liquid and dry, these models were done at the lab to be evaluated against the strept equi sub species equi. Every day every model is testes against the strept bacterial count, for three successive days.

In liquid form the disinfectants was diluted using tap water.in semiliquid form the disinfectant was mixed with surfactant (pril) in a ratio 2:3 (disinfectant: pril respectively).

In the liquid and semiliquid model: The disinfectant is mixed with water or surfactant till the initial concentration of the active principle of each disinfectant become 5%for all disinfectants except virkon 2%.

In the Dry form: The disinfectant was mixed with lime till the initial concentration of the active principle of each disinfectant become 5% for all disinfectants except virkon 2%.

Samples from different disinfectant foot baths (1ml for liquid and semiliquid and 1 g for dry) before and after dipping of the coupons for one minute contact were taken daily for three successive days and added to 9 ml neutralization tube mixed by vortex and left at 20˚c for 5 min neutralization. Then ten- fold serial dilution and agar inoculation were performed.

Surface disinfection tests: A film of bacterial cells was placed onto the surface of a coupon (rubber) and after 1 hour drying at 37 C, the coupon was dipped into100ml of the disinfectant for a specified contact time (one minute).this step was repeated daily for three successive days, The number of surviving bacteria in each coupon was determined by standard culture plate and incubation .according to European Committee for standardization (2001): European standard EN13697: (EN 13697) Quantitative Surface Test of Bactericidal Activity). Total *strept equi* count is done at the lab using specified media.

RESULTS AND DISCUSSION

The result of microscopic examination showing, irregularly shaped gram positive cocci. The bacterium also causes red blood cells to lyse (β-haemolytic colonies). It is catalase-negative. The tested strain was identified genotypically by PCR which revealed that a positive amplification of 196 bp fragment of primer specific for *tuf* gene specific for the genus *streptococci* and positive amplification of 110 bp fragment of primer *eqbe* gene specific to *S. equi* subsp. *equi*.

The antimicrobial sensitivity test of the tested strain of *S. equi subsp. equi* in Table 3 showed antibiotic resistance against tetracycline, cortimoxazole, rifampicin and cephalothin while it showed susceptibility to vancomycin, erythromycin, clindamycin and chloramphenicol. *S. equi* is highly resistant to phagocytosis, which means that infection can be established despite the abundance of neutrophils and other factors of the innate immunity., although recent studies suggests that shedding may last for several months (Gröndahl *et al.*, 2012).

Ensuring you have good biosecurity measures and procedures in horse place will ensure your horse stays safe and well,Disinfectant foot bath outside isolation stables and at the yard entrance was recommended to prevent strangles infection. An important component of any biosecurity programs is controlling the mechanical transmission of pathogens by staff members through the strategic placement of foot baths and disinfectant mats.

In our work, we studied the effect of five disinfectants used as foot bath in three models (liquid and semiliquid and dry) footbaths to evaluate their effects against *sreptococcus equi sub species equi* isolated from native horse breed in Egypt.

The results in Table 4, 5 and 6 showed the count log of *strep.equi* of different models of foot bath before and after one minute of application of the contaminated rubber coupons in the foot bath for three successive days to study the ability of footbath to withstand the continuous bacterial load from contaminated shoes day after day, our results showed increasing the count of *strept.equi* day after day as the count log was increased from zero to 3, 4.3,5.3, 2 and 4.66 for Ca hypochlorite 5%, Halamid 5%, Staldaren 5%,,Virkon S 2% and Formalin 5% respectively in case of liquid footbath. And reached 3.3, 3.04, 6.66, 3 and 6.4 for Ca hypochlorite 5%, Halamid 5%, Staldaren 5%, Virkon S 2% and Formalin 5% respectively in case of semiliquid footbath and finally the strept equi count reached 5.7, 5.57,3.4, 5.7 and 6.6 for Ca hypochlorite 5%, Halamid 5%, Staldaren 5%,Virkon S 2% and paraformaldehyde 5% respectively in case of dry footbath.

Ca hypochlorite 5% and Virkon S 2% in their liquid and semiliquid form showed the lowest count after 48 hours, Ehsan et al. 2017, attributed, the increased count day after day when using Ca hypochlorite 5% to the decrease in chlorine concentration gradually day after day which consequently decrease the effectiveness of the disinfectant

Virkon S was approved as a bactericidal, virucidal, fungicidal and sporicidal agent for use in foot bath (DuPont 2010).

Staldren 5% was better in dry footbath than the other two types, Halamid 5% showed the lowest count log in semiliquid foot bath, and finally formalin5% showed the highest count log in all types of foot baths used specially in dry form.

Table 1: Oligonucleotide primers sequences source: Metabion (Germany).

Gene	Sequence	Amplified product	Reference
Streptococcus tuf gene	GTACAGTTGCTTCAGGACGTATC ACGTTCGATTTCATCACGTTG	196 bp	Picard *et al*., 2004
S. equi subsp. Equi eqbe gene	ATGTAGCTATGGCAAATGTGGC CAGGTGTTCCTAAGGGTGTT	110 bp	North *et al*., 2014

Table 2: The disinfectant agents used

Chemical	Active chemical	Manufacture
Pril @	Non-ionic surfactants (5%) and anionic surfactants (5-15%).	Henkel factory production (Made in Egypt)
Calcium Carbonate	Ca2Co3	Egyptian company for chemicals production
Commercial Calcium hypoclorite powder	Chlorine conc. 89%	Egyptian company for chemicals production
Halmid	Chlorine coc. 24.4%	A product by axcentive sarl, your dedicated partner for desinfection
Staldren	Chloramine percentage is 10 %	A product from J.N. Jorenku
Virkon s	Potassium peroxymonosulfate.....21.41% Sodium Chloride.....1.50% Other ingredients .....77.09% Total ingredient 100.00%	A product by DuPont
Formalin solution	Formalin 38%	A product by Loba chemie
Paraformaldehyde powder	Paraformaldehyde 95%	A product by Loba chemie

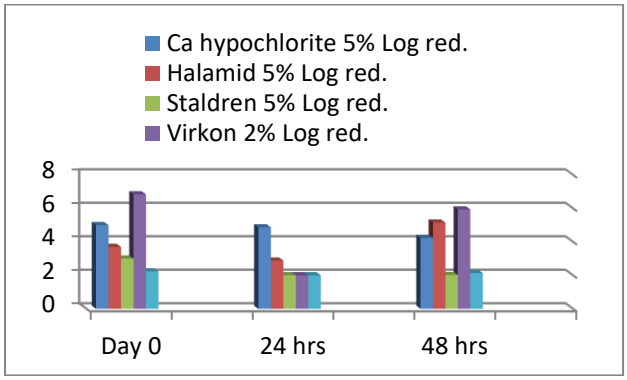

Fig. 1: Strept equi count Log reduction of coupons before and after 1min contact in liquid foot bath for three successive days

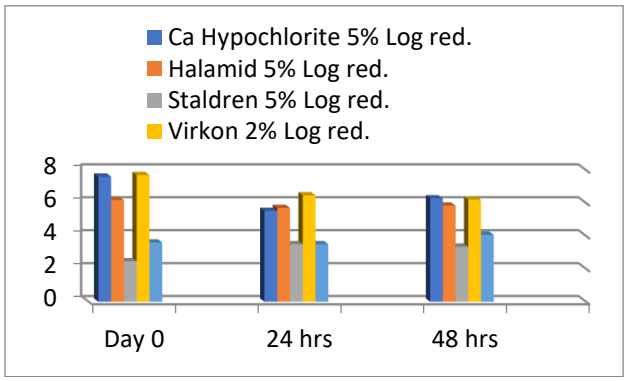

Fig. 2: Strept equi count Log reduction of coupons before and after 1min contact in semiliquid foot bath for three successive days.

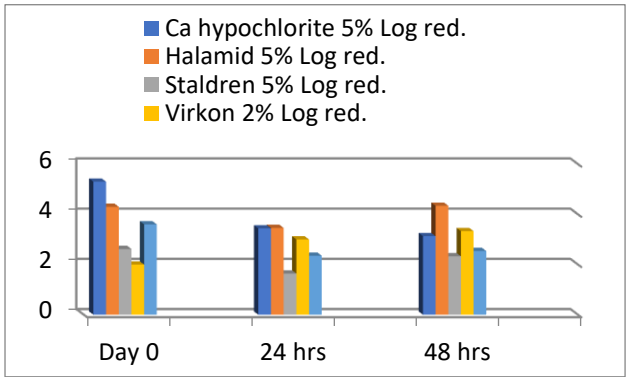

Fig. 3: Strept equi count Log reduction of coupons before and after 1min contact in dry foot bath for three successive days.

Table 3: Antibiogram assay of *S. equi subsp. equi* isolated from native equine breed

Antimicrobial agent		R	I	S
Tetracycline	TE	R		
Vancomycin	VA			S
Erythromycin	E			S
Clindamycin	DA			S
Cortimoxazole	SXT	R		
Rifampicin	RD	R		
Cephalothin	KF	R		
Chloramphenicol	C			S

Ehsan *et al.*, 2017 found that, the concentration of chlorine in staldren not affected at the third day of experiment in all types of footbath but begin to decrease from the 10th day in case of dry form. Surviving of bacteria in liquid disinfectant bath highlights the opposite role of the liquid foot bath in preventing spread of disease in poultry farms (shimaa *et al.*, 2018).

Many researches indicated that wrongly managed footbath will no longer serve as a preventive biosecurity measure but will be consider as tool of disease (Heinzel &Bellinge 1982) cleared that, foot bath disinfectants, in the worth case could represent a contamination problem with resistant, biofilm forming bacteria that may be transferred to the production area by the footwear. Bacterial growth in aqueous solution of disinfectants has been reported and foot baths could thus serve as a contamination source.

Quinn (2001) stated that, Boot baths should be refilled at least every 2-3 days and for best results, it should be replaced daily.

Regarding *strept equi* count Log reduction of coupons before and after 1min contact in different types of footbath bath for three successive days Table 7, Fig. (1) showed that, the highest reduction was achieved when we used virkon S 2% as it was still having bactericidal effect even after 48 hrs of the experiment (log.red, 5.94)

Amass *et al*., 2005 cleared that, dipping clean boots in a boot bath of 1 % Virkon S was effective. and was found that when the soles of footwear were dried after treatment with Virkon®, there was a significant reduction in bacterial numbers.

In Halamid 5%, the log reduction begun with 3.7 and then decreased to 2.8 after 24 hours and then increased again to reach 5.15 after 24 hours it may be due to the evaporation of water led to this phenomenon (Ehsan 2017). Parkar *et al*. 2004 approved that, Halamid has a bactericidal effect against thermophilic bacilli.

Ca hypochlorite 5% showed bacterial reduction at the zero day as it reached 5 log reduction but after that the reduction decreased to 4.24 after 48 hours. Diluted bleach and foot baths contaminated by organic debris quickly become inactivated (Dwyer R, 2004).

The bacterial reduction was reduced when we used staldren 5 % (3.02) followed by formalin 5% (2.014) in liquid form foot bath.

Disinfectant-filled mats may not be effective in reducing the bacterial load on floors or in reducing mechanical tracking of S enterica from contaminated areas in a veterinary teaching hospital. Hartmann et al., 2013.

Regarding semiliquid footbath Table 8, Fig. 2 cleared that, the semiliquid foot bath showed the best results as the reduction was improved for all types of disinfectants used that showed bacterial log reduction >5 except formalin (4.04) and staldren (3.35).

Both Ca hypochlorite 5% and Verkon S 2%% achieved good bactericidal activity as they reached 6.28 for ca.hypochlorite and 6.2 for verkon S 2% after 48 hours of application followed by Halamid 5% (5.84), the concentration of chlorine in halamid 5% semiliquid foot bath was increased after 48 hours of application to reach 6.5% (Ehsan 2017). These results may be due to decrease the amount of water that will decrease the bacterial growth within the bath and also increase the contact between the microorganism and disinfectant and prolong the action of disinfectant used.

Table 4: Strept equi count log of liquid foot bath before and after 1min contact for three successive days

Time	Disinf.	Ca hypochlorite 5%	Halamid 5%	Staldren 5%	Virkon 2%	Formalin 5%
Day 0	before	*Zero*	*Zero*	*Zero*	*Zero*	*Zero*
	After	*Zero*	*Zero*	4.7	1	1.9
24 hrs	before	2.77	3.6	4.7	1.6	3.6
	After	2.77	4.3	5.8	2.2	3.8
48 hrs	before	3	3.6	5.2	1.6	3.3
	After	3	4.3	5.3	2	4.66

Table 5: Strept equi count log of semiliquid foot bath before and after 1min contact for three successive days

Time	Disinf.	Ca hypochlorite 5%	Halamid 5%	Staldren 5%	Virkon 2%	Formalin 5%
Day 0	before	*Zero*	*Zero*	*Zero*	*Zero*	*Zero*
	After	3.77	3.17	3.6	2.2	2
24 hrs	before	zero	3.38.	3.07	*zero*	4.3
	After	3	4.3	4.47	3.5	5.3
48 hrs	before	3.11	3	5	1.6	5.4
	After	3.3	3.04	6.66	3	6.4

Table 6: Strept equi count log of dry foot bath before and after 1min contact for three successive days

Time	Disinf.	Ca hypochlorite 5%	Halamid 5%	Staldren 5%	Virkon 2%	Paraformaldehyde 5%
Day 0	before	*Zero*	*Zero*	*Zero*	*Zero*	*Zero*
	After	3	4	5.6	3.77	5.68
24 hrs	before	3.6	4	4.07	2	3.6
	After	3.9	3.77	4.6	3.6	6.6
48 hrs	before	2.3	3.3	2.6	1.6	3.6
	After	5.7	5.57	3.4	5.7	6.6

Table 7: Strept equi count Log reduction of coupons before and after 1min contact in liquid foot bath for three successive days

	Disinf	Ca hypochlorite 5%		Halamid 5%		Staldren 5%		Virkon 2%		Formalin 5%	
		log	Log red.	log	Log red.	log	Log red.	log	Log red.	log	Log red.
Day	before	8	5	7.6	3.7	8.9	3	8.44	6.84	8.09	2.24
0	After	3		3.9		5.9		1.6		5.85	
24	before	8.47	4.87	7.95	2.88	8	2	6.77	2	8.32	2
hrs	After	3.6		5.07		6		4.77		6.3	
48	before	8.38	4.24	8.14	5.15	8.32	2.02	8.34	5.94	8.34	2.14
hrs	After	4.14		3		6.3		2.4		6.2	

Table 8: Strept equi count Log reduction of coupons before and after 1min contact in semiliquid foot bath for three successive days

Time	Disinf.	Ca hypochlorite 5%		Halamid 5%		Staldren 5%		Virkon 2%		Formalin 5%	
		log	Log red.	log	Log red.	log	Log red.	log	Log red.	log	Log red.
Day 0	before	8.9	7.6	9.38	6.18	9.07	2.46	9.3	7.7	9.6	3.6
	After	1.3		3.2		6.61		1.6		6.0	
24 hrs	before	9.07	5.52	9.25	5.7	9.2	3.5	9.77	6.47	9.8	3.5
	After	3.55		3.55		5.7		3.3		6.3	
48 hrs	before	9.66	6.28	9.14	5.84	9.6	3.35	9.5	6.2	9.07	4.07
	After	3.38		3.3		6.25		3.3		5.0	

Table 9: Strept equi count Log reduction of coupons before and after 1min contact in dry foot bath for three successive days

Time	Disinf	Ca hypochlorite 5%		Halamid 5%		Staldren 5%		Virkon 2%		Paraformaldehyde 5%	
		log	Log red.	log	Log red.	log	Log red.	log	Log red.	log	Log red.
Day	before	7.6	5.3	7.6	4.3	8.0	2.62	7.6	2.0	7.6	3.6
0	After	2.3		3.3		5.38		5.6		4.0	
24	before	8.44	3.44	8.6	3.46	8.54	1.64	8.34	3.0	8.9	2.35
hrs	After	5.0		5.14		6.9		5.34		6.55	
48	before	8.6	3.13	8.84	4.34	8.47	2.33	8.9	3.33	8.44	2.54
hrs	After	5.47		4.5		6.14		5.57		5.9	

The strong inhibitory effect of ca hypochlorite appeared to be due to a higher pH (Kang *et al.* 2013), Cheah *et al.*, (2009) considered Virkon® as a standard biosecurity treatment for disinfecting footwear.

Formalin 5% in semiliquid form was somewhat better than in liquid form as the log bacterial count was 4.07 after 48 hours, staldren 5% produced 2.04 log reductions at the zero day then the reduction increased to 3.5 and 3.35 after 24 and 48 hours respectively.

The semisolid foot baths need much time to act, but this did not lessen its powerful capacity to remain clean for 3 successive days than the liquid baths (Shimaa *et al.*, 2018).

On the other hand, Table 9, Fig. 3 cleared that, all used disinfectants were not powerful in the dry form as compared with liquid and semi liquid form,the bacterial log reduction decreased gradually from the first day to reach 3.13, 4.34, 2.33, 3.33 and 2.54 for Ca hypochlorite 5%, Halamid 5%, staldren 5%, VirkonS 2% and paraformaldehyde 5%.

These results may be due to the decrease of concentration of chlorine by time when used in dry form

of foot bath.The concentration of chlorine in ca hypochlorite 5% dry foot bath decreased day after day (Ehsan, 2017).

The cleaning and sanitation of studied items contaminated with S. equi is generally effective, however, it is material dependent and cleaning protocols should be adjusted to different material. (Ryden *et al*., 2017).

The use of footbaths and foot mats containing effective disinfectants may help decrease the risk for spread of nosocomial infection but should not be expected to sterilize footwear. (Dunowska, *et al*., 2006).

Conclusion

The footbath as a simple form of biosecurity is usually soiled with microorganism and should be frequently replaced to avoid to be useless or to be a source of infection specially in the liquid form, semiliquid foot bath prolonged the action of disinfectant when used and produce excellent bactericidal activity when compared with liquid and dry form.

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