Journal of Equine Veterinary Science 121 (2023) 104204

Contents lists available at ScienceDirect

Journal of Equine Veterinary Science

journal homepage: www.j-evs.com

Effectiveness of Cleaning and Sanitation of Stable Environment and Riding Equipment Following Contamination With *Streptococcus equi* Subsp. *equi*

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ARTICLE INFO

Article history:
Received 26 October 2022
Received in revised form 22 December 2022
Accepted 26 December 2022
Available online 29 December 2022

Keywords:
Horse
Strangles
Washing machine
Leather
Polyester halter

ABSTRACT

Streptococcus equi subsp. *equi* (*S. equi*) is transmitted via contact with infected horses or fomites such as equipment or surfaces of the stable environment. Effective cleaning and sanitation is essential to minimize risk of fomite-associated infections. This study assessed the effectiveness of cleaning and sanitation of experimentally *S. equi* contaminated materials and equipment found in stables. Wood, concrete, plastic, leather halters, leather gloves and polyester webbing halters were inoculated with a 24-hour culture *S. equi* laboratory strain. In addition, selected materials were inoculated with a clinical strain of *S. equi*. Three days post inoculation all materials were sampled for retention of viable *S. equi* and a subset of each material was cleaned and sanitized. After an additional 2 days all treated and untreated materials were sampled for continued retention of viable *S. equi*. Separate subsets of contaminated polyester halter material were washed at 40°C with or without drying at 70°C, or washed at 60°C. After cleaning and sanitation, all samples except polyester halters were culture negative. Even before cleaning and sanitation leather appears to poorly support survival of *S. equi*. After washing at 40°C and tumble drying, 14 of 16 halters were culture positive, however culture negative when washed at 60°C. Routine cleaning and sanitation of fomites contaminated with *S. equi* was generally effective to eliminate viable bacteria. However, survival between materials and strains differed, with leather poorly permissive to *S. equi* survival even without cleaning, whereas polyester webbing halters retained viable *S. equi* even after washing at temperatures of 40°C.

1. Introduction

S. equi is the causative agent for the upper respiratory disease "strangles" in horses [1,2]. The bacteria is transmitted directly by contact with infected horses or indirectly via contaminated equipment such as training equipment, stall/,pasture objects and water buckets [3,4]. Under laboratory conditions, *S. equi* survives on wood

Conflict of interest statement: The authors declare no potential conflict of interest with respect to the research, authorship, and/or publication of this article.

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Date: 20221026

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https://doi.org/10.1016/j.jevs.2022.104204

for 63 days at 2°C and on glass and wood for 48 days at 20°C [3]. In field conditions on similar materials *S. equi* appeared to survive from 34 days [5] up to 72 days [6] whereas in later summer conditions outdoors with exposure to sunlight and high temperatures, survival was limited to 1–3 days [7].

Cleaning of equipment and stables should include physical removal of visible organic material followed by use of an appropriate disinfectant proven to act against *S. equi* [8]. Among commonly used disinfectants for stables, povidone iodine, chlorhexidine gluconate and peroxide-containing compounds have been proven to destroy the organism within 90 minutes, whereas phosphoric acid and sodium hypochlorite, diluted according to the manufacturer's recommendation, are poorly effective [6]. During the past decade, strangles research has focused largely on improving strangles diagnostics and understanding of disease mechanisms; in particular spread of strangles by silent carriers [9,10]. However, the roll continued spread of disease by contaminated inanimate objects and fomites in the stables with strangles outbreak is poorly understood [11]. Consequently, there is a pressing need to identify whether, following strangles outbreaks, viable *S. equi* can be eliminated from the horse's environment, including stall, and riding/training equipment.

The aim of this study was to determine whether cleaning and sanitation procedures according to a commonly used protocol on broth contaminated stall materials and horse equipment could effectively eliminate recovery of viable *S. equi* from their surfaces. Additionally we assessed whether material type and treatment influenced bacterial survival.

2. Material and Methods

2.1. Bacterial Inoculum

Two reference laboratory strains (*Streptococcus equi subsp. equi* CCUG 23255 and ATCC 33398) (LS1 and LS2) and one nontyped clinical strain (CS) of *S. equi*, isolated from the guttural pouch in a silent carrier horse, were used in this study. The strains were prepared by adding two colonies of *S. equi* strain into 250 ml brain heart infusion broth (BHIB, CM 1135, Thermo Fisher Scientific, Uppsala, Sweden), incubated for 20 hours at 37°C, and then plated according to standard laboratory procedures. The colony-forming unit (CFU) concentrations of *S. equi* in the inoculums were 2.1 x 10^8 CFU/mL in the first batch (LS1), 5.3 x 10^8 CFU/mL in the second batch (LS2) and 1.8 x 10^8 CFU/mL in the CS batch.

2.2. Inoculation of Environment and Equipment

During the study, biosafety level 2 according to Directive 2000/54/EC, was used to protect personnel and surrounding environment to exposure to the biological agent. The study was performed indoors in a separate isolation facility held at 10°C– 18°C and without exposure to direct sunlight. To illustrate bacterial contamination from horses to stable environment and riding equipment, 24 pieces (5 x 5 cm) of outdoor concrete tiles, individual pieces of rough sawn untreated wooden boards, plastic water buckets, leather halters (3 x 5 cm), leather gloves, polyester webbing halters (3 x 5 cm) were inoculated with LS1 inoculum batch (2.1 x 10^8 CFU/mL). Furthermore, 24 pieces (5 x 5 cm) of individual pieces of rough sawn untreated wooden boards, plastic water buckets, leather halters (3 x 5 cm) and newly manufactured, ecological tanned, leather halters (3 x 5 cm) were inoculated with CS inoculum batch (1.8 x 10^8 CFU/mL). As subsequent results showed continued recovery of viable *S. equi* on polyester webbing halters despite cleaning and disinfection an additional 24 pieces of this material were inoculated using LS2 broth batch (5.3 x 10^8 CFU/mL) and subjected to alternate cleaning methods. Inoculation of all materials was performed by pouring 4.5 mL of the *S. equi* broth on each piece, after which the broth was dispersed over the entire surface. All material treated, except newly manufactured leather halters had been used for several years in a stable environment.

2.3. Cleaning and Sanitation

The protocol for cleaning and sanitation is presented in Fig. 1. Briefly, 3 days after inoculation, each group of material was sampled to confirm bacterial growth postcontamination and of the remaining unsampled pieces, half were subjected to cleaning and sanitation according to recommendations from the national veterinary institute (SVA, Uppsala, Sweden) and the remainder retained as uncleaned controls. For the sanitation group, each item was thoroughly scrubbed for 1 minute with lukewarm water and detergent containing alcohol ethoxylate 15% (Fri Ren Natur, Nilfisk, Brondby, Denmark), left for 10 minutes and then rinsed with tap water. After 2 hours, each item was soaked in a disinfectant containing potassium monopersulfate, maleic acid and sulfamic acid (DesiDos SeptiChem ApS, Holte, Denmark) and left undisturbed for 2 days to dry (Fig. 1A and B). The 24 additional pieces of polyester halter webbing (Fig. 1C) were left for 3 days after inoculation. Sixteen of these were then washed in a commercial washing machine (Electrolux W4130H) set at 40°C for 39 minutes with an alkaline cleaner (Rekolex FL-10, Rekal Svenska AB, Gnesta, Sweden). After washing, 8 pieces were left to air dry, whereas 8 others were placed in a tumble drier (Electrolux Line 6000) set at 70°C until the items were dry (60 minutes). The remaining 8 pieces of polyester were washed in the washing machine set at 60°C for 43 minutes. After washing, all 24 pieces of halter were left for 2 days until bacterial sampling. After the study was completed, the washing machine was run several times set at 90°C without any items in the machine to prevent bacterial contamination. When plastic materials were contaminated with the CS, a disinfectant containing oxyacid of chlorine (ECO horse Removeit, Scandinavian AB, Sweden) was used and evaluated.

2.4. Bacterial Sampling

Bacterial sampling was performed by brushing the entire surface of each piece of material for 1 minute with sterile cloths (SodiBox, Nevéz, France) intended for microbiological surface sampling in food production. The cloths were premoistened with a buffered peptone solution containing a 10% additive of a neutralizing substance (lecitine, Tween 80, L-histidine and sodiumthiosulphate). After sampling, the cloths were placed in individual sterile plastic bags, and immediately processed in the laboratory for culture.

2.5. Culture and Analysis

Buffered peptone water (100 mL) was added to each bag with the cloth and rotated rapidly for 2 minutes using a Stomacher labblender (easyMIX Lab Blender, AES-Chemunex, Weber Scientific). The resulting fluid was transferred from the plastic bag to 50 mL plastic tubes (Sarsted), and centrifuged at 3000 x g for 10 minutes. One µl of the pellets was collected and plated on agar plates with 5% defibrinated horse blood, supplemented with colistin acid and oxalinic acid (COBA plates). All culture plates were then incubated in 5% CO_2 atmosphere in 37°C and resulting numbers of CFU of *S. equi* was classified positive ($\geq$ 1 CFU) or negative after 24 and 48 hours. One colony was selected from each group and analyzed with matrix assisted laser desorption ionization-time of flight mass spectrometry MALDI-TOF (Bruker Nederland BV, Leiderdorp, Netherlands) for microbiological identification.

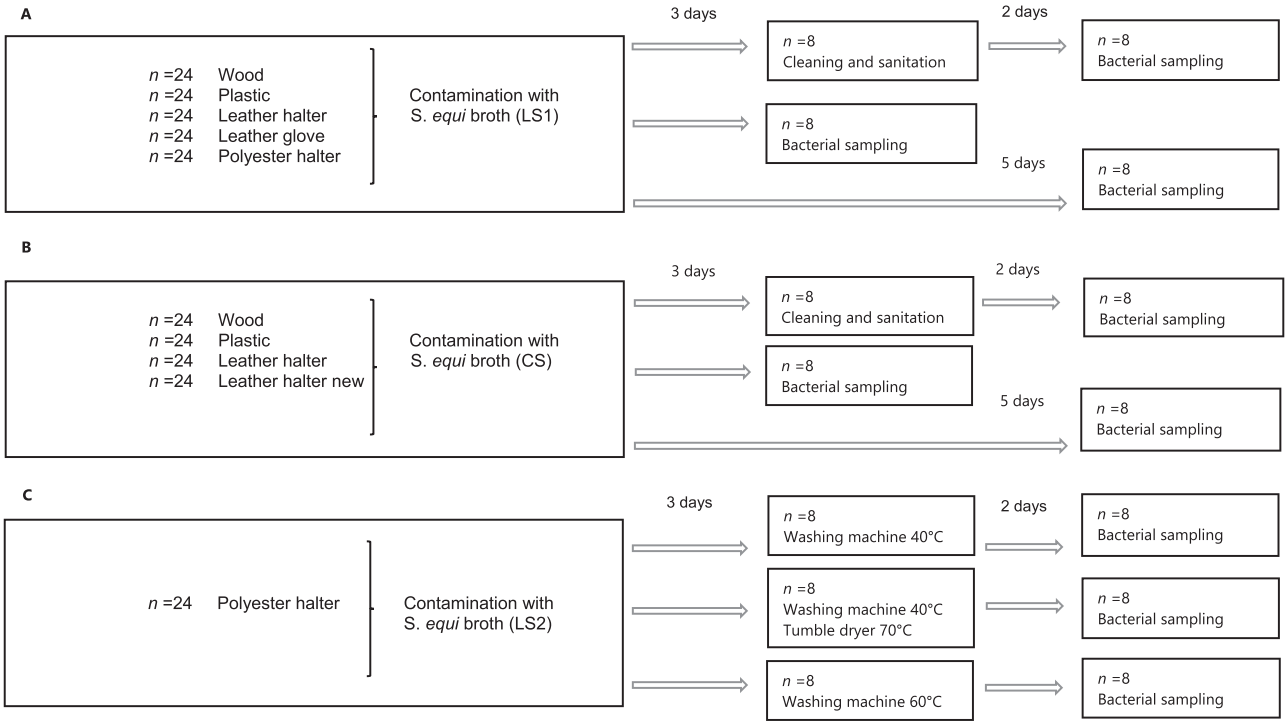

Fig. 1. Protocol for cleaning, sanitation and bacterial sampling on pieces of selected stable environment and riding equipment contaminated with *S. equi* of laboratory strains (LS1 and LS2) and clinical strain (CS).

Table 1
Survival of *S. equi* of laboratory strain (LS1) on inoculated material and for selected materials inoculated with clinical strain (CS) after cleaning and sanitation compared to controls.

Material	Control 3d	Control 5d	After cleaning and sanitation	$P =$
Concrete LS1	8[a]/8[b]	8/8	0/8[c]	.0002
Concrete CS	-	-	-	
Untreated wood LS1	8/8	8/8	0/8[c]	.0002
Untreated wood CS	8/8	7/8	0/8[c]	.0014
Plastic LS1	8/8	8/8	0/8[c]	.0002
Plastic CS	8/8	8/8	0/8[c]	.0002
Leather halter LS1	4/8	1/8	0/8 ns	1
Leather halter CS	8/8	7/8	0/8[c]	.0014
Leather halter new CS	8/8	1/8	0/8 ns	1
Leather gloves LS1	7/8	0/8	0/8 ns	1
Leather gloves CS	-	-	-	
Polyester halter LS1	8/8	8/8	6/8 ns	.4667
Polyester halter CS	-	-	-	

Abbreviation: ns, not significant.
[a] Number of samples from which *S. equi* was grown.
[b] Total numbers of samples tested.
[c] $P < .05$ Fishers exact.

2.6. Statistical Methods

Comparison of recovery versus nonrecovery of *S. equi* over time after 3 days, after 5 days and after cleaning to controls (no cleaning) of all materials was assessed using the Fisher's exact test for categorical data with significance set at $P < .05$ on a one tailed test.

Table 2
Survival of *S. equi* of laboratory strain (LS2) on inoculated polyester halter after cleaning in a commercial washing machine.

Material	Washing machine 40°C	Washing machine 40°C + tumble dryer	Washing machine 60°C
Polyester halter	7[a]/8[b]	7/8	0/8 ($P < .001$)

[a] Number of samples from which *S. equi* was grown.
[b] Total numbers of samples tested.

3. Results

3.1. Survival of S. equi on Different Materials

The survival of the three *S. equi* strains on the different materials is shown in Tables 1 and 2.

After cleaning and sanitation of materials contaminated with LS1 or CS, all samples from each material were culture negative for *S. equi*, apart from the pieces of polyester halter, of which 6 of 8 remained culture positive. Whereas control samples from concrete, wood (7/8), plastic, and polyester halter were culture positive 3 and 5 days after contamination, only 4 of 8 control samples from leather halters contaminated with LS1 were culture positive after 3 days, and 5 days after contamination only 1 of 8 of controls

was culture positive. Similarly, 7 of 8 samples from leather gloves initially culture positive after 3 days had become culture negative by 5 days post despite lack of cleaning and sanitation. When materials were contaminated with CS, all control samples were culture positive 3 days after contamination whereas 7 of 8 samples from concrete, 7 of 8 samples from leather halters but only 1 of 8 control sample from new leather halters were culture positive 5 days after contamination. For the procedure of the additional pieces of polyester halter 14 of 16 items were culture positive for *S. equi* after machine washing at 40°C, and 7 of 8 remained culture positive after washing at 40°C followed by tumble drier at 70°C. However, after washing at 60°C all (8/8) halter material pieces were culture negative (Table 2).

4. Discussion

The results of the present study suggest that routine methods for manually cleaning and sanitation was appropriate to rid horse stables of viable *S. equi* from most of the commonly utilized materials. Additionally, it appears that, even without cleaning and sanitation, leather products commonly used in horse sports in most cases fail to support longer-term survival of *S. equi*. On the other hand, woven polyester from halters appears to retain viable *S. equi* despite routine cleaning or even washing at 40°C and tumble drying.

4.1. Survival of S. equi Before Cleaning and Sanitation

Curiously, survival of *S. equi* on leather halters varied between strains. When inoculated with LS1, leather from both leather halters and leather gloves, even without cleaning and sanitation appeared to poorly support survival of *S. equi*, whereas the CS demonstrated longer survival time on the used leather halters. However, as only one sample collected from new leather halters contaminated with CS was culture positive 5 days postcontamination the difference may have been related to different structure of the surface or amount of dirt on the two different halters used. Variation between survival of bacteria of laboratory and field strains of *S. equi* has been described earlier [11]. In leather production, selected substances used in the tanning process, such as sulphide-lime liquors and chromium [12] or chromium-free ecological alternatives [13] that transform animal hides to preserved leather products have an adverse effect on growth of microorganisms [14,15]. Consequently, residues of products with antimicrobial effects used in the tanning process may have been a key reason for the poor survival of *S. equi* on the leather materials.

4.2. Cleaning and Sanitation Methods

In view of the variety of surfaces that may be contaminated in horse stables, sanitation of equine premises following infectious diseases poses a challenge. Sanitation involves use of various chemical disinfectants. Selection is dictated by the type of material and rigor of sanitation required but also avoid substances toxic to horses and staff. Evidence based guidelines on optimal cleaning and sanitation in veterinary settings are uncommon and recommendations in literature are often based on trial and error [16]. The cleaning and sanitation methods used in our experiment were used in either field practice or in equine hospitals. While surfaces best suited for successful disinfection are nonporous materials, such as stainless steel, glass and smooth enamel-painted surfaces, equine stables are commonly constructed with concrete and unfinished wood with porous and rough surfaces that represent a challenge to cleaning and sanitation. However, the results in our study demonstrate that routine cleaning and sanitation methods eliminates viable *S. equi* even from such rough surfaces. On the other hand, similar routine cleaning and sanitation appeared ineffective in eliminating viable *S. equi* from polyester halters. The most plausible reason for this finding was that halters are a woven fabric that facilitate absorption of contaminating fluid media and subsequent retention of moisture, creating a favorable environment for bacterial survival. Reported survival of microorganisms on textiles after machine washing varies widely [17–19] with no apparent agreement on methodology such as laundering temperature and duration. Importantly, laundering procedures that eliminate viable microorganisms are dependent on several factors including type of material, duration of washing cycle, mechanical action of laundering procedure, dosage and type of added detergents [20]. For example, Walter and coworkers reported that *Staphylococcus aureus* on linen survived a 10 minutes laundering at 54°C followed by drying, but could be eliminated with a 60°C laundering procedure [19]. Further, textiles used in health care are to be washed at high temperatures and include detergent containing activated oxygen bleach [21] to reduce potential infection risks.

By extension our polyester halters remained culture positive at 40°C even with subsequent tumble drying at 70°C, whereas simply washing at 60°C rids the halters of viable *S. equi*.

A key drawback of this study was that survival of *S. equi* on stall materials and fomites was examined indoors under constant room temperature conditions, whereas in field conditions horse stalls are subject to a far wider variation in temperatures that may also influence bacterial survival following a strangles outbreak. Moreover, it remains to be determined whether the bacterial load in our experimental contamination by live *S. equi* approximated the bacterial load contamination of fomites during a strangles outbreak, and if the presence of additional body secretions in field settings influence overall bacterial survival.

4.3. Culture and Analysis

Based on initial findings it was determined that sample dilution was not performed as the aim was to maximize the detection of bacteria numbers remaining on the materials after various control or cleaning procedures, and that results were classified solely as positive or negative.

5. Conclusions

It appears that routine cleaning and sanitation of most materials found in equine stables can effectively eliminate contamination by viable *S. equi*. Importantly however, materials such as polyester webbing halters require machine washing at 60°C to eliminate viable *S. equi*. On the other hand, even without rigorous cleaning leather appears to poorly support survival of *S. equi*, which has key consequences for managing and care of leather bridles and saddles. Incorporation of findings from this work in directed sanitation after a strangles outbreak can assist in prevention of spread to new animals via fomites and stall materials.

Acknowledgment

We are grateful to John Pringle for his critical reading of the manuscript. The excellent assistance during the experiment by veterinary nurse students Anneli Nilsson, Elin Johansson and Erika Nilsson is much appreciated.

Financial disclosure

This study was funded by grants from the Swedish Research Council (Formas) (2019-02127).

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