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Antibacterial Efficacy of Some Antiseptics and Disinfectants against Common Bacterial Agents Isolated from Horses in Turkey

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Abstract

Nowadays, many disinfectants and antiseptics are used for decontamination purposes in equine hospitals, on racetracks, and breeding farms, but generally, these antimicrobial agents are not tested against commonly encountered pathogens, and they are used with unknown antimicrobial efficacy. The antimicrobial efficacies of ethanol, chlorhexidine, povidone iodine, sodium hypochloride, peroxymonosulfate compound, and benzalkonium chloride were analyzed using the quantitative suspension test method against the field isolates of *Escherichia coli, Pseudomonas aeruginosa, Salmonella* spp., *Streprococcus zooepidemicus, Streptococcus equi, Rhodococcus equi,* and *Staphylococcus auerus,* which are the most frequently encountered pathogens of equines, in the presence of organic load (10% fetal bovine serum) after 1 min, 5 mins, and 30 mins contact times at 20°C. A log reduction of five or more (5 log ≤) in cfu counts of the tested pathogens was considered as effective for each disinfectant and antiseptic. According to the results, except for sodium hypochloride in the 1/100 dilution, all other disinfectants and antiseptics achieved a minimum 5 log reduction and were found to be effective against all tested isolates. Decreased dilutions and/or direct use of the sodium hypochloride should be tested against the same bacterial agents, as well as with multiple field strains. In addition, reference strains of the microorganisms should be evaluated in further studies.

Keywords: Antiseptics, bacterial pathogens, biosecurity, disinfectants, horse

Introduction

Control of infectious diseases in horse populations involves two critical aspects: vaccination and disinfection. Many adequate vaccines against infectious diseases are commercially available, but none of them can be warranted to be 100% effective (Dwyer, 2004). In times of an epidemic disease, it is common to find significant environmental microbial contamination in hospitals, on racetracks, farms, and in any facilities where horses reside. This microbial contamination commonly orginates from infected animals' secretions, such as blood, urine, feces, nasal, and conjunctival secretions, etc. (Saklou et al., 2016). It is also important to minimize animal trafficking and distribution of potential pathogens by movement of personnel and fomites (Morley et al., 2005). Therefore, disinfection and antisepsis management practices are essential parts of providing a healthy environment for horses. Disinfection also plays an important role in the prevention and control of nosocomial infections, especially for the multi-resistant bacteria, where disinfection is the only way to slow down the disease outbreak.

Many bacterial pathogens can cause systemic and local infections in horses. *Streptococcus equi* subsp. *zooepidemicus* (*S. zooepidemicus*) and *S. equi* subsp. *equi* (*S. equi*) cause the lower respiratory tract, joint, genital tract, eye and guttural pouch infections, and abscess formation. In foals, *Rhodococcus equi* (*R. equi*) cause pleuropneumonia, gastrointestinal tract infections, and abscess formation as well. *Staphylococcus aureus* (*S. aureus*)

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may cause wound infections, mastitis, and abscess formation. *Salmonella* spp. are mostly isolated from the gastrointestinal tract infections and neonatal sepsis. *Escherichia coli* (*E. coli*) may cause genital tract infections, mastitis in mares, and septicemia in neonatal foals. *Pseudomonas aeruginosa* (*P. aeruginosa*) cause genital tract infections and mastitis in mares (Sellon and Long, 2013). These bacterial agents can survive on environmental surfaces for long periods with a possible transmission to susceptible hosts. Therefore, it is imperative to use an effective disinfectant/antiseptic to prevent the spread of these agents (Köse and Yapar, 2017).

In field conditions, a good disinfectant should be effective in the presence of organic matter, such as blood, urine, feces, and other body secretions; have a low or zero toxicity against animals; and show the bactericidal activity in a relatively short period of time. Among the horse pathogens, gram-positive and gram-negative bacteria and enveloped viruses are considered to be susceptible to the disinfectants in the absence of organic load. But besides these generalizations, because they are in the same susceptibility category, *Salmonella* species are extremely difficult to eliminate from horse facilities (Dwyer, 2004).

In Turkey, many commercially available antiseptics and disinfectants with different active ingredients are used in equine industry for decontamination of the bacterial agents. But to the best of author's knowledge, no antimicrobial efficacy studies with commercially available antiseptics and disinfectants were performed against the reference and field strains of the horse bacterial pathogens up to this date in Turkey.

The aim of this study was to evaluate the antibacterial effectiveness of disinfectants and antiseptics often used in equine facilities and hospitals, including sodium hypocloride (household bleach), potassium peroxymonosulfate (Virkon S; İstanbul, Turkey), and benzalkonium chloride (Quaternary ammonium compound-QAC, Zefirolum; İstanbul, Turkey) as disinfectants as well as ethanol, povidone iodine (Poviiodeks; İstanbul, Turkey), and chlorhexidine (Hibitanol; İstanbul, Turkey) as antiseptics against the field isolate of gram-positive species such as *S. zooepidemicus*, *S. equi*, *S. aureus*, and *R. equi*, and gram-negative species such as *P. aeruginosa*, *E. coli,* and *Salmonella* spp. in the presence of organic load to examine the antimicrobial activities of the commercial compound(s) commonly used in horse care facilities and hospitals in Turkey.

Table 1. Sample type isolated, date of isolation, and geographical isolation region of the bacterial isolates used in the study

Isolate	Sample type isolated	Geographical origin
Gram positive		
S. zooepidemicus	Tracheal wash fluid	İstanbul
S. equi	Guttural pouch wash fluid	İstanbul
S. aureus	Skin wound swab	İstanbul
R. equi	Tracheal wash fluid	İzmit
Gram negative		
E. coli	Intrauterine swab	Thrace
P. aeruginosa	Intrauterine swab	Thrace
Salmonella spp.	Rectal swab	İstanbul

Materials and Methods

Bacterial Strains

The field isolates of *S. zooepidemicus*, *S. equi*, *S. aureus*, *R. equi*, *E. coli*, *P. aeruginosa*, and *Salmonella* spp. were used in the study. The isolation side, date of isolation, and isolation region in the country were shown in the table below (Table 1). Briefly, all clinical samples were streaked to 5% sheep blood agar and MacConkey agar, and they were incubated at 37°C in both aerobic and microaerophilic (5% CO_2) conditions for 48 hours. In addition, rectal swab samples were also inoculated in selenite broth for 18 hours and passaged to the XLD agar media for *Salmonella* spp. isolation. Suspected colonies were identified with routine methods, such as colony morphology, microscopic morphology, and gram characteristics, catalase, oxidase, and other biochemical tests using the BBL crystal E/NF and gram-positive identification systems (Becton Dictinson; Sparks, U.S.). After the identification process, the isolates were passaged into tryptone soy broth (TSB) (Oxoid; Basingstoke, UK), containing 20% glycerol, and stored at −20°C until laboratory analysis. The isolates were revived by passaging to the tryptone soy agar (TSA) (Oxoid; Basingstoke, UK) from the storage media.

Disinfectants and Antiseptics

Commercially available three different classes of disinfectants and three different classes of antiseptics were chosen to represent different range of active compounds in the present study. Disinfectant/antiseptic classes, active ingredients, and the dilutions used in the experiment are given in Table 2. Disinfectants and ethanol were diluted using tap water of 207 mg $CaCO_3$/L hardness in each test tube. The hardness of the tap water was determined with SM 2340:B; ISO 17294-2 (ICP-MS) method by a private enviromental analysis laboratory (Çevre Industrial Analysis Laboratory, İstanbul, Turkey).

Culture Media

Tryptone soy broth and TSA were used for maintenance and determination of viable cell counts in the experiment.

Neutralization Media

Neutralization solution was prepared by using a mixture of tryptone (5.0 g/L), yeast extract (2.5 g/L), dextrose (10.0 g/L), sodium thioglycollate (1.0 g/L), sodium thiosulphate (6.0 g/L), sodium bisulphite (2.5 g/L), llecithin (7.0 g/L), polysorbate 80 (5.0 g/L), and bromocresol purple (0.020 g/L). The final pH values of neutralization media were measured and adjusted to 7.6±0.2 before use.

Table 2. Name, class, active ingredients, and dilutions of the disinfectants and antiseptics used in the study

Disinfectant/antiseptic name	Disinfectant/antiseptic class	Active ingredient	Used dilution
Virkon-S	Peroxygen compounds	Potassium peroxymonosulfate (50%)	1:100
Zefirolum	QAC	Benzalkonium Chloride (10%)	1:100
Household bleach	Chlorine compounds	Sodium hypochloride (5.25%)	1:100
Ethanol	Alcohols	Ethanol (70%)	Direct use
Poviiodeks	Iodine compounds	Povidone iodine (10%)	Direct use
Hibitanol	Biguanides	Chlorhexidine (4%)	Direct use

Organic Load
Fetal bovine serum (FBS) at a final solution of 10% was used in the experiment.

Contact Time
Contact times of 1 min, 5 mins, and 30 mins at 20°C for each disinfectant/antiseptics against the bacterial suspensions were included in the experiment.

Experiment Control Procedures
Three control procedures were performed to demonstrate the validity of the experiment. Standard tap water that was used in the dilution of the antiseptics and disinfectants was controlled for the lethal effect against bacterial growth. Bacterial growth for all microorganisms tested in the study was determined in TSA after a 24-hour incubation at 37°C. The neutralizan solution used in the study was also checked for the lethal effect on the bacterial growth.

Standard tap water was used in the dilution of the antiseptics/disinfectants and controlled for the lethal effect against bacterial growth. For the evaluation, 1 mL of bacterial suspension and 1 mL of organic substance were added to 8 mL of tap water instead of disinfectant/antiseptic and then incubated for 5 minutes at room temperature. After incubation, 0.1 mL of the incubated suspension was inoculated into TSA and incubated at 37°C for 24 hours and checked for bacterial growth.

The neutralizan solution used in the study was controlled for the lethal effect against bacterial growth. For the evaluation; 8 mL of neutralizan solution and 1 mL of sterile distilled water were added to 1 mL of bacterial suspension and then incubated for 5 minutes at room temperature. After incubation, 0.1 mL of the incubated suspension was inoculated to TSA and incubated at 37°C for 24 hours and checked for bacterial growth.

The effect of the neutralizan solution for each antiseptics/disinfectant used in the study was checked. For control, 1 mL of bacterial suspension and 1 mL of sterile distilled water were added to 8 mL of neutralized disinfectant solution and then incubated for 5 minutes at room temperature. After incubation, 0.1 mL of the incubated suspension was inoculated to TSA and incubated at 37°C for 24 hours and checked for bacterial growth.

Test Method
The effectiveness of the disinfectants was evaluated by the method of quantitative suspension test (Ismail et al., 2015). Broth cultures of bacterial strains were stored at −20°C until the experiments. Cultures were brought to room temperature, and then 0.1 mL of broth cultures were inoculated to TSA, being allowed to grow at 37°C for 24 hours. A subculture was performed for each bacterial strain, and the second subcultures of the bacterial strains were used in the study. All suspensions from the second subcultures were prepared with TSB and adjusted to $1.5x10^8$ cfu (colony forming unit)/mL by plate surface spread viable counting method. The bacterial suspensions were maintained at room temperature and used within 2 hours.

Prior to testing, all reagents were brought to 20°C in water bath. Disinfectant/antiseptics were diluted with tap water as recommended by the manufacturers. 1 mL of FBS solution was added to 8 mL disinfectant/antiseptic solution and mixed by vortexing and left for 30 minutes. 1 mL of bacterial suspension was added to the mixture and inoculated at 20°C for 1 min, 5 mins, and 30 mins, respectively. After contact time of the bacterial strain with the disinfectant/antiseptic solution, 1 mL of disinfectant/antiseptic+bacterial strain mixture was added to 8 mL of neutralization media with 1 mL sterile distilled water and inoculated for 5 minutes at 20°C.

After the neutralization step, 100 µl of mixture was inoculated to the TSA with serial dilutions up to 10^{-5} at 37°C for 18 hours to determine cfu counts.

Reduction of viability of the microorganisms were calculated according to the following formula:

$$R = \frac{N \times 10^{-1}}{Na}$$

where R is the reduction in viability, N is the cfu count of the initial test suspension, and Na is the cfu count of the mixture at the end of the contact time with the disinfectant/antiseptic suspension.

A minimum log reduction of 5 (5 log≤) was defined as effective for the disinfectants/antiseptics used in the study.

Table 3. Cfu/mL values of *E. coli* after contact with tested antiseptics and disinfectants at 20°C

Antiseptics-disinfectants/contact time	1 min	5 mins	30 mins
Ethanol	0	0	0
Chlorhexidine	0	0	0
Povidone iodine	0	0	0
Sodium hypochloride	$1.04x10^5$	$6x10^4$	$7x10^0$
Virkon S	0	0	0
Benzalkonium Chloride	0	0	0

Table 4. Cfu/mL values of *P. aeruginosa* after contact with tested antiseptics and disinfectants at 20°C

Antiseptics-disinfectants/contact time	1 min	5 mins	30 mins
Ethanol	0	0	0
Chlorhexidine	0	0	0
Povidone iodine	0	0	0
Sodium hypochloride	$5.4x10^4$	$7x10^4$	$3x10^4$
Virkon S	0	0	0
Benzalkonium Chloride	0	0	0

Table 5. Cfu/mL values of *Salmonella* spp. after contact with tested antiseptics and disinfectants at 20°C

Antiseptics-disinfectants/contact time	1 min	5 mins	30 mins
Ethanol	0	0	0
Chlorhexidine	0	0	0
Povidone iodine	0	0	0
Sodium hypochloride	$8.75x10^3$	$2.2x10^4$	$2.6x10^4$
Virkon S	0	0	0
Benzalkonium Chloride	0	0	0

Table 6. Cfu/mL values of *S. zooepidemicus* after contact with tested antiseptics and disinfectants at 20°C

Antiseptics-disinfectants/contact time	1 min	5 mins	30 mins
Ethanol	0	0	0
Chlorhexidine	0	0	0
Povidone iodine	0	0	0
Sodium hypochloride	$4x10^1$	0	0
Virkon S	0	0	0
Benzalkonium Chloride	0	0	0

Table7. Cfu/mL values of *S. equi* after contact with tested antiseptics and disinfectants at 20°C

Antiseptics-disinfectants/contact time	1 min	5 mins	30 mins
Ethanol	0	0	0
Chlorhexidine	0	0	0
Povidone iodine	0	0	0
Sodium hypochloride	$2.02x10^4$	$1.92x10^4$	$1.17x10^4$
Virkon S	0	0	0
Benzalkonium Chloride	0	0	0

Table 8. Cfu/mL values of *R. equi* after contact with tested antiseptics and disinfectants at 20°C

Antiseptics-disinfectants/contact time	1 min	5 mins	30 mins
Ethanol	0	0	0
Chlorhexidine	0	0	0
Povidone iodine	0	0	0
Sodium hypochloride	$2.31x10^3$	$1.9x10^4$	0
Virkon S	0	0	0
Benzalkonium Chloride	0	0	0

Results

Experiment Control

Test results indicated that no antibacterial effect of the neutralizan solution was determined for all tested microorganisms in the study. The neutralizan effect of the neutralizan solution against antiseptics/disinfectants was evaluated for each disinfectant and antiseptic as well. No inhibition in bacterial growth was determined for each neutralized disinfectant and antiseptic used in the study (data not shown).

Antibacterial Activity of the Tested Antiseptics and Disinfectants

According to the standards, animicrobials tested must show a minimum 5 log (10^5) reduction in cfu/mL to be considered as effective. After the determined contact times with 70% ethanol, chlorhexidine, povidone iodine, virkonS (1/100), and benzalkonium chloride, a 8.17 log reduction was identified against *E. coli*, *P. aeruginosa*, *Salmonella* spp., *S. zooepidemicus*, *S. equi*, *R. equi*, and *S. aureus* in the presence of the organic load (10% FBS). But on the other hand, sodium hypochloride (1/100) failed to pass the test standard against *E. coli* after 1 min and 5 mins; against *P. aeruginosa* after 1 min, 5 mins, and 30 mins; against *Salmonella* spp. after 1 min, 5 mins, and 30 mins; against *S. equi* after 1 min, 5 mins, and 30 mins; against *R. equi* after 1 min and 5 mins; and against *S. aureus* after 1 min and 5 mins contact times in the presence of organic load (10% FBS). The cfu/ml of bacterial agents after 1 min, 5 mins, and 30 mins contact times with the antiseptics and disinfectants are listed as in the tables (Tables 3-9). Results of reduction in viabilities (log reduction) obtained in the present study were given as graph (Figure 1).

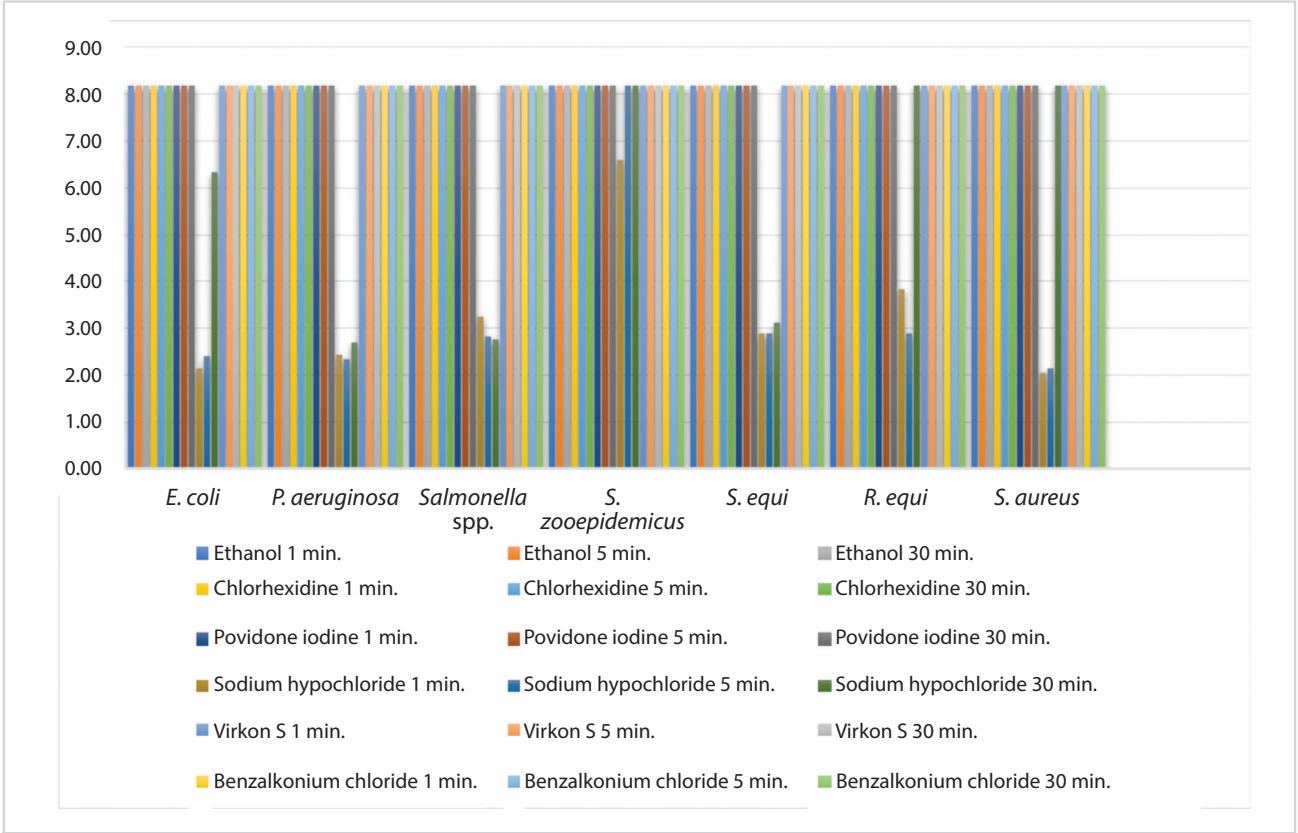

Figure 1. Results of reduction in viability (log reductions) of bacterial isolates against tested antiseptics/disinfectants

Table 9. Cfu/mL values of *S. aureus* after contact with tested antiseptics and disinfectants at 20°C

Antiseptics-disinfectants/contact time	1 min	5 mins	30 mins
Ethanol	0	0	0
Chlorhexidine	0	0	0
Povidone iodine	0	0	0
Sodium hypochloride	$1.39x10^5$	$1.11x10^5$	0
Virkon S	0	0	0
Benzalkonium Chloride	0	0	0

Discussion

The use of disinfectants and antiseptics is of paramount importance in biosecurity and infection control in individuals and populations. Proper use of disinfectants and antiseptics could be expected to be cheaper than economic cost of antimicrobial treatment in horses or loss of part or all of that horse population due to a disease outbreak (Dwyer, 1995). Microorganisms are known to vary in their susceptibility against disinfectants and antiseptics, and some studies reveal that the efficacy of disinfectants are gradually reduced (Orji, 2014). Inappropriate consumption, inaccurate concentration, and lack of training for preparation and storage are the most common reasons of increasing resistance to disinfectants (Zareniya et al., 2017).

Karayıldırım and Çelenk (2016) expressed that 20% benzalkonium chloride was found to be effective against *E. coli*, *S. aureus,* and *P. aeruginosa* with a 1 min contact time. In the present study, it was determined that the 1/100 dilution of 10% benzalkonium chloride was also effective (log 5 ≤ reduction) in 1 min, 5 mins, and 30 mins against the same bacteria that were isolated from clinical cases of horses. Gehan et al. (2009) indicated that 1% of benzalkonium chloride was effective against *P. aeruginosa*, *E. coli*, *S. typhimurium,* and *S. aureus* at the 30 min contact time. In another study, 3% of benzalkonium chloride achieved a 5 log reduction in 30 mins, and 1% in 60 mins (El Aal et al., 2008). Fazlara and Ekhtelat (2012) described that *Listeria monocytogenes* was the most susceptible bacteria to benzalkonium chloride, followed by *S. aureus* and *E. coli*, respectively, according to the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) test results. Considering all these results, in addition to 20% after 1 min of contact time, 1% and 3% concentration of benzalkonium chloride after 30 mins of contact time, and 1/100 dilution of %10 benzalkonium chloride can be used for inactivating *E. coli*, *S. aureus,* and *P. aeruginosa* after 1 min, 5 min, and 30 mins contact time.

Saklou et al. (2016) determined that the mist application of 2% of peroxymonosulfate compound disinfectant at 30 mins contact time created a 84%, 99%, and 99% reduction against *S. enterica*, *P. aeruginosa,* and *S. aureus,* respectively, and it was found to be effective if used after cleaning of the surfaces. Gehan et al. (2009) found that 1% peroxymonosulfate compound was effective against *P. aeruginosa*, *E. coli*, *S. typhimurium,* and *S. aureus* after 30 mins of contact time in the presence of organic matter. Chima et al. (2013) also declared that peroxymonosulfate compound was 100% effective against *Salmonella* spp., *E. coli*, *Klebsiella* spp., and *P. aeruginosa*. Another study also yielded that 1/100 dilution of 50% peroxymonosulfate compound demonstrated 5 log ≤ reduction against *S. typhimurium* ATCC 13311 strain (Jang et al., 2017). These results were in concordance with the present study's results: 50% peroxymonosulfate disinfectant in 1/100 dilution showed a 8.17 log reduction at 1 min, 5 mins, and 30 mins, and it was found to be effective (5 log ≤) for all pathogens that participated in the study and found to be effective against the same bacterial pathogens of the previous studies. The present study's results for the peroxymonosulfate compound have been also confirmed in previous studies.

In the present study, 5.25% sodium hypochloride in 1/100 dilution failed to create the 5 log ≤ reduction against *P. aeruginosa* at 1 min, 5 mins, 30 mins and *S. aureus* at 1 min and 5 mins of contact time. But contrary to the present study, 5% sodium chloride in 1/100 dilution showed 7.22 and 8.11 log reductions at 5 mins and 30 mins, respectively, in a previous study (Bhosale, 2017). The discrepancy might have been due to the difference of the antimicrobial resistancy of bacterial strains tested in the studies. On the other hand, Addie et al. (2015) specified that household bleach produced a 5 log ≤ reduction in *E. coli* 0157:H7 and *Salmonella typhimurium* after 1 min contact time. In the present study, the household bleach was used in 1/100 dilution so that the efficacy might have been reduced due to the dilution factor. In parallel, Avcı and Otkun (2017) claimed that the 1/100 dilution of sodium chloride was ineffective against *S. aureus* and *P. aeruginosa* after 1 min and 2 mins contact time but effective after 5 mins, 10 mins, and 30 mins contact time. According to the author, further studies should be designed to test more concentrated dilutions or direct use of the sodium hypochloride against the same pathogens in to test the antimicrobial efficacy. It was also claimed that sodium hypochloride was inactivated by organic debris (Addie et al., 2015). Another reason for reduction in the efficacy of sodium hypochloride against the tested pathogens might have been the interaction with organic material (10% FBS) in the present experiment.

Zareniya et al. (2017) determined that povidone iodine was more effective than 70% ethanol in 49 *P. aeruginosa* isolates according to MIC and MBC values. In the present study both antiseptics demonstrated a full reduction (8.17 log) in *P. aeruginosa*. In another study, 10% povidone iodine was found to be effective after 1 min contact time against *S. aureus*, *P. aeruginosa*, and *E. coli* (Avcı and Otkun, 2017). In the same study 70% ethanol was effective against *P. aeruginosa* and *E. coli*, but just ineffective against *S. aureus* after 1 min contact time. After 2 mins, 5 mins, 10 mins, and 30 mins, 70% ethanol was found to be effective by Avcı and Otkun (2017). The present study revealed that 70% ethanol and 10% povidone iodine demonstrated efficacy (5 log ≤) against *P. aeruginosa*, *S. aureus*, and *E. coli* after 1 mins, 5 mins, and 30 mins contact time, and the results were mostly in concordance with Avcı and Otkun's (2017) results. According to the past and present study results, 70% ethanol and 10% povidone iodine can be used for antisepsis and disinfection purposes. The present study has some limitations, such as limited number of field bacterial isolates were tested, and a single test method (quantative suspension test) was used. Therefore, future studies should include more of field strains and evaluate different efficacy methods to monitor the antimicrobial activity of the tested disinfectants.

In conclusion, 50% peroxymonosulfate compound in 1/100 dilution; 10% benzalkonium chloride in 1/100 dilution as disinfectants; and 70% ethanol, 4% chlorhexidine, and 10% povidon iodine as antiseptics may be used in equine hospitals and equine care facilities as decontaminating agents against *E. coli*, *P. aeruginosa*, *Salmonella* spp., *S. zooepidemicus*, *S. equi*, *R. equi*, and *S. aureus* after 1 min, 5 mins, and 30 mins contact time. Sodium hypochloride in 1/100 dilution did not yield satisfactory results, and it failed to achieve a 5-log reduction against most of the bacterial agents tested in the study. The dilution ratio of sodium hypochloride may be decreased or used without diluting while testing against the bacterial agents in further studies. The present study tested one field isolate of each bacterial species against disinfectants and antiseptics as representatives. Future comprehensive studies should also be performed with multiple field and reference strains of *E. coli*, *P. aeruginosa*, *Salmonella* spp., *S. zooepidemicus*, *S. equi*, *R. equi,* and *S. aureus,* as well as other bacterial and mycotic pathogens with different analytical methods to evaluate the antimicrobial efficacies of the disinfectants and antiseptics.

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