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Suitability of two commercial preparations for disinfections against methicillin-resistant *Staphylococcus aureus* in veterinary medicine

Eignung von zwei Handelspräparaten zu Desinfektionen gegen Methicillin-resistenten Staphylococcus aureus in der Veterinärmedizin

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Summary

Two commercially available preparations from the disinfectants list of the German Veterinary Association (Deutsche Veterinärmedizinische Gesellschaft e. V. (DVG)) were tested against a methicillin-resistant *Staphylococcus aureus* (MRSA) field isolate in a concentration as recommended in the list for disinfection of bacteria. The "Guidelines of DVG for testing of disinfection methods and chemical disinfectants" were used as methodical base. Initially, the examinations for determination of the minimal inhibitory concentrations (MIC) and suitable inactivation substances were carried out. The bactericidal effect of both commercial preparations – a formic acid preparation (55 g per 100 g) and a preparation containing 21 g glutaraldhyde plus 17 g per 100 g formaldehyde – as well as reference disinfectants (37% formaldehyde and 99.5% phenol) were examined in suspension and carrier tests. The suspension tests were performed without and with protein load (20% FBS). Pieces of linden wood were used as carriers.
The recommendation of DVG for both Venno® Vet 1 super and M&enno® Veterinär B neu as bactericide (except of bacterial spores) is 30 min (for preventive disinfections) as well as 120 min contact (for specific disinfections) of 1% concentration. The results show that the disinfection of MRSA do not require higher concentrations and longer contact times as recommended in the disinfectant list of DVG for both commercial disinfectants. Both products were effective in suspension tests in a 1% concentration and within five minutes independent of protein load. In contrast, 1% phenol (reference disinfectant for suspension tests) needed 30 min reaction time without protein load as well as 60 min with protein load for complete inactivation of the MRSA field strain. In carrier tests, a 30 min contact of 1% concentration of both commercial disinfectants and 3% concentration of formalin (reference disinfectant for carrier tests) was sufficient for complete disinfection.

Keywords: animal hygiene, disinfectant test, disinfection, methicillin-resistence, *Staphylococcus aureus*, MRSA

Zusammenfassung

Zwei Handelspräparate aus der Desinfektionsmittelliste der Deutschen Veterinärmedizinischen Gesellschaft e.V. (DVG) wurden in einer Eckwertprüfung auf Bakterizidie gegen ein Methicillin-resistentes *Staphylococcus aureus* (MRSA) Feldisolat getestet. Als methodische Basis dienten die „Richtlinien der DVG für die Prüfung von Desinfektionsverfahren und chemischen Desinfektionsmitteln". Demnach wurden zunächst Untersuchungen zur Bestimmung der minimalen Hemmkonzentration (MHK) und zur Ermittlung geeigneter Inaktivierungsmittel durchgeführt. Die bakterizide Wirkung beider Präparate (ein 55 g/100 g Ameisensäure enthaltendes Mittel und ein 21 g/100 g Glutaraldehyd plus 17 g/100 g Formaldehyd enthaltendes Präparat) sowie die Referenzdesinfektionsmittel, bestehend aus 37 % Formaldehyd und 99,5 % Phenol p.a., wurden in Suspensions- und Keimträgertests geprüft. Suspensionsversuche wurden ohne und mit Proteinbelastung (20 % FKS) durchgeführt. Als Keimträger wurden Stückchen aus Lindenholz verwendet.
Die Anwendungsempfehlung der DVG für die beiden Präparate, Venno® Vet 1 super und M&enno® Veterinär B neu, als bakterizid (mit Ausnahme von Bak-

Zusammenfassung

teriensporen) ist 30 min (zur vorbeugenden Desinfektion) bzw. 120 min Einwirkung (zur speziellen Desinfektion) der 1%igen Konzentration. Die Ergebnisse zeigen, dass die Desinfektion des getesteten MRSA-Feldisolats keine höheren Konzentrationen und keine längeren Einwirkzeiten bedingt, als sie in der DVG-Liste für die beiden Handelspräparaten angegeben sind. Die beiden Produkte sind wirksam in Suspensionstests mit und ohne Proteinbelastung in einer Konzentration von jeweils 1 % innerhalb von fünf Minuten. Im Gegensatz dazu benötigt 1 % Phenol (Referenzdesinfektionsmittel für Suspensionsversuche) eine Kontaktzeit von 30 Minuten ohne Proteinbelastung bzw. 60 Minuten mit Proteinbelastung für die vollständige Inaktivierung des MRSA-Feldisolates. 30 Minuten Einwirkung beider kommerziellen Präparate in einer Konzentration von 1 % führte zur vollständigen Desinfektion der Keimträger. Auch die 3%ige Lösung des Formalins (Referenzdesinfektionsmittel für Keimträgerversuche) desinfizierte die Keimträger innerhalb von 30 Minuten.

Schlüsselwörter: Tierhygiene, Desinfektionsmittelprüfung, Desinfektion, Methicillin-Resistenz, *Staphylococcus aureus*, MRSA

Introduction

The genus *Staphylococcus* belongs to the family *Staphylococcaceae*. Staphylococci are gram-positive, facultative anaerobic, spherical cells of 0.5–1.5 µm in diameter. Some of the *Staphylococcus* species are confined to a few hosts, whereas others, particularly *S. aureus*, have a wide host spectrum (Selbitz, 2007). Most of the staphylococcal species belong to the normal flora of humans und animals (Walther et al., 2006) and colonize mucosal membranes and the outer skin (Selbitz, 2007). Species such as *S. aureus*, *S. epidermidis*, *S. intermedius*, *S. hyicus* are considered as facultative pathogens. Under promotive conditions like immunosuppression, poor hygiene and organ lesions they can lead to local or systemic disease (Walther et al., 2006). Signs may range from mild skin alterations to life-threatening bacteraemia (Leonard and Markey, 2008).

In the past, staphylococci were generally sensitive against β-lactam antibiotics, erythromycin, lincomycin, gentamicin, fluorfenicol and fluorquinolones (Selbitz, 2007). However, owing to the *mec*A gen, methicillin-resistant *Staphylococcus aureus* strains (MRSA) display resistance against all penicillins, cephalosporins, and carbapenems. Beyond these, they are often resistant against additional anti-infective drugs i.e. aminoglycosides, macrolides, lincosamide, streptomycine, tetracyclines, chloramphenicol, fluorquinolones and rifampicin (Walther et al., 2006).

MRSA colonise not only humans but also animals and are therefore a major and increasing problem in the fields of veterinary medicine. Since the first isolation of MRSA from milk of mastitic cows (Devriese et al., 1972), MRSA were detected in several other domestic animals like dogs, cats, horses, sheep, pigs and chickens (Leonard and Markey, 2008). Transmissions of MRSA between humans and animals are very likely (Weese et al., 2006, Meemken et al., 2008). After a meta-analysis of several epidemiological studies by Salgado et al. (2003), the prevalence of MRSA colonisation among persons without health care-associated risk factors is clearly lower (0.2%) than average MRSA colonisation prevalence (1.3%). The data on the prevalence among veterinarians and veterinary personal are even more alarming. A study concerning 272 veterinarians, which have regular contacts to livestock of pigs, showed that 12.5% of the humans carry MRSA (Wulf et al., 2008). Hanselman et al. (2006) indicated that veterinary personal working with large animals have more frequently colonisation (15 of 96, 15.6%) than personal employed in small animal practice (12 of 272, 4.4%) or those without animal patient contacts (0 of 50). In an other study, a MRSA prevalence of 4,6% was detected among 152 veterinarians and veterinary students in professional contact with livestock (Wulf et al., 2006).

The reduction of the bacterial load by proper cleansing and disinfection in animal houses can minimize indirect transmissions animal-to-human, human-to-animal and animal-to-animal. Therefore, it is necessary to determine whether the disinfectants used in the veterinary area are also suitable for preventive and specific disinfection in cases of MRSA contaminations in animal houses. In this study we determined the efficacy of two commercially available chemical disinfectants from list of DVG using a MRSA isolate under standardized laboratory conditions in suspension and germ carrier tests.

Materials

Test organism

Methicillin-resistant *Staphylococcus aureus* represents a clinical isolate from a dog with arthritis, Isolate-Nr: 10698. The isolate was obtained as broth culture from Prof. Dr. Lothar H. Wieler, DVM, Dipl. ECVPH, Institute for Animal and Environmental Hygiene, Veterinary Faculty, Free University Berlin.

Culture Media

Soybean-casein digest broth, SCB (BBL Tripticase Soy Broth, Becton, Dickinson and Company, Sparks, USA).

Tryptone soya agar, TSA (CASO-Agar, Oxoid, Wesel, Germany).

Inactivation substances

1. 0.1 mol/l in disodium hydrogen phosphate in SCB
2. 0.2 mol/l disodium hydrogen phosphate in SCB
3. 3% polysorbat 80 + 0.3% lecithin + 3% saponin + 0.1% histidin in SCB
4. 3% polysorbat 80 + 0.3% lecithin + 3% saponin + 0.1% histidin + 0.3% sodium thiosulfate in SCB

5. 3% polysorbat 80 + 0.3% lecithin + 3% saponin + 0.1% histidin + 0.3% sodium thiosulfate + 0.01 mol/l disodium hydrogen phosphate in SCB
6. 1% polysorbat 80 in SCB

Substance for protein load

Foetal bovine serum, FBS (PAA Laboratoires, Pasching, Austria), inactivated at 56°C for 30 min.

Diluents

Hard water according DVG: 17,5 ml of 10% (w/v) $CaCl_2.6\ H_2O$ + 5 ml of 10% (w/v) $MgSO_4.7\ H_2O$ in 3300 ml Aqua dest.

Trypton-NaCl solution: 1.0 g caseinpepton + 8.5 g NaCl in 1000 ml Aqua dest.

Commercial disinfectants

Venno® Vet 1 super (Menno Chemie, Norderstedt, Germany): 55 g/100 g formic acid

M&enno® Veterinär B neu (Menno Chemie, Norderstedt, Germany): 21 g/100 g glutaraldehyde, 17 g/100 g formaldehyde

Reference disinfectants

Formalin (formaldehyde 37%, VWR International, Leuven, Belgium).

Phenol (pro analysi, 99.5%, Merck, Darmstadt, Germany).

Carriers

Sterilized pieces of untreated linden wood, 1 cm^2 x 3 mm.

Methods

All tests were performed according to the guidelines of the German Veterinary Association (Deutsche Veterinärmedizinische Gesellschaft e. V., DVG, Giessen) (Anonymous, 2007a).

Production of the suspension of the test organism

The test organism was subcultured two times at 37°C for 18 h (stock culture). From the stock culture a subculture (working culture) was prepared in the same way. After determination of colony forming units (cfu) on TSA using the spread plate technique, the number of bacterial cells was adjusted to 1 x 10^8 to 1 x 10^9 cfu/ml by dilution with SCB.

Determination of the minimal inhibitory concentration (MIC)

The disinfectants were diluted using hard water. As control served a dilution series of phenol. Each of 5 ml of disinfectant solution in stepped concentrations was mixed with 5 ml of SCB in 2 x concentration. These were inoculated with 0.1 ml of 10 x diluted working culture. After 72 h of incubation at 37°C the tubes were checked for turbidity. The cloudy tubes indicate multiplication of test organism. The lowest concentration that had inhibited multiplication was assessed as MIC. For the control of multiplication, subcultures are set onto TSA from cloudy tubes on limit range.

Determination of suitable inactivation substances

The examinations were carried out as described above, but using inactivation substances (see material: the given concentrations correspond to the end concentrations).

Suspension tests

0.1 ml of working culture was mixed with 2 ml hard water (suspension test without protein load) or 2 ml inactivated FBS (suspension test with protein load). Subsequently, 8 ml disinfectant dilution (in 1.25 x of desired test concentration) was added. After contact times 5, 15, 30 and 60 min at 20°C samples of 0.1 ml were transferred into the tubes with 10 ml SCB containing inactivation substances. Tubes were checked for turbidity after 72 h incubation at 37°C. As control 1 hard water, and as control 2 phenol in a concentration of 1% was used.

Carrier tests

Carriers were dipped in working culture for 20 sec, subsequently placed on blotter paper and dried at room temperature for about 30 min. Following, they were dipped in 20 ml of disinfectant solution for 2 min (contact time) and placed into Petri dishes in vertical position (reaction time). After 30, 60, 120 min they were transferred into tubes with 10 ml SCB containing inactivation substances. After 72 h incubation at 37°C tubes were checked for turbidity. As control 1 hard water and as control 2 formalin in a concentration of 3% was used. For exact determination of final value and identification of test organism, subcultures are set onto TSA from cloudy tubes at limit range. The tubes without turbidity were inoculated with 0.05 ml of working culture in order to control residual disinfectant effect.

Results

Determination of minimal inhibitory concentration (Tab. 1)

The formic acid containing product Venno® Vet 1 super inhibit multiplication of MRSA at concentrations of 0.016% and higher. The MIC of M&enno® Veterinär B neu is 0.125%. Also in 0.25% phenol, no multiplication of the MRSA strain could be determined. The subcultures from cloudy tubes onto TSA result already after 24 h of incubation in the appearance of characteristic colonies (small, round, convex, gray-white colonies with smooth surface and range).

Determination of suitable inactivation substances (Tab. 1)

The multiplication was inhibited by 0.016% Venno® Vet 1 super without inactivation substances. The best inactivation of the disinfectant is achieved using inactivation substance 5. M&enno® Veterinär B neu showed a MIC of 0.125% without inactivation substances. None of the inactivation substances tested was able to eliminate the inhibiting effect of 0.25% M&enno® Veterinär B neu. The control substance phenol had a bacteriostatic effect at 0.25%. The combinations 3 and 4 proved to be suitable as inactivation substances for phenol. Further examinations in suspension and carrier tests with Venno® Vet 1 super, M&enno® Veterinär B neu and formalin were carried out using inactivation substance 5. For phenol the inactivation substance 4 was used.

Results of suspension tests (Tab. 2)

Both, Venno® Vet 1 super and M&enno® Veterinär B neu in a concentration of 1% disinfected MRSA within 5 min independent of protein load. A 30 min reaction of 1% phenol lead to inactivation of MRSA in suspension without protein load. The suspension with 20% FBS (test under protein load) could be disinfected after 60 min effect of phenol.

Results of carrier tests (Tab. 3)

The tubes of control 1 were cloudy in both test sets. No multiplication of MRSA could be detected after 30 min reaction of 1% Venno® Vet 1,1% M&enno® Veterinär B neu and 3% formalin. The tubes without turbidity, which were inoculated with 0,05 ml of working culture, shoved already after 24 h incubation turbidity that indicated the absence of residual disinfectant effect. The subculture from control 1 onto TSA resulted in formation of characteristic colonies of the test organism.

Discussion

Resistance is either a hereditary natural property of an organism or acquired by mutation or acquisition of plasmids or transposons (McDonnell and Russell, 1999). The widespread use of antibacterial drugs in human and veterinary practice resulted in the development of resistant bacteria of many species including staphylococci (Inoue et al., 1998, Sørum and Sunde, 2001, Schwarz and Chaslus-Dancla, 2001, Collignon, 2002). Reports concerning increased resistance to antiseptics and disinfectants are also numerous. Irrational use of antimicrobial drugs as well as biocides (needless use, incorrect choice, low dosage, short contact, irregular application) is mostly responsible for the emergence of resistant strains. Kirchhoff (1962) and Wille (1976) showed experimentally that the exposure to sub lethal concentrations of a chemical disinfectant by repeated sub-passages can result in the development of resistance within a bacterial population. Genetic studies provided evidence that the acquired methicillin-resistance is due to the incorporation of the mecA gene into the genome of staphylococci (Selbitz, 2007). It is currently unknown if the presence of the mecA gene is simultaneously associated with enhanced resistance of staphylococci against chemical disinfectants. *S. aureus* strains with plasmid gene encoding resistance to gentamicin tolerate some antiseptics and disinfectants like chlorhexidine, diamidines, and quaternary ammonium compounds better (McDonnell and Russell, 1999). Mycock (1985) indicates a very significant increase in tolerance of MRSA to povidone-iodine. Also Cookson et al. (1991) showed that possession of certain plasmids, which are responsible for resistance to nucleic acid binding components (e. g. ethidium bromide), determine the MIC of chlorhexidine to MRSA.

S. aureus can very well adhere to hydrophobic surfaces like plastic and stainless steel (Götz, 2002). Because of high tenacity of MRSA in arid conditions, it can survive from weeks to months on non-nutritive inanimate surfaces as well as in dried organic material (Smith et al., 1996; Neely and Maley, 2000; Wagenvoort et al., 2000; Sexton et al., 2006; Kramer et al., 2006). The usual conditions in animal houses benefit adhesion, survival and even multiplication of bacteria. The construction, surface qualities of the used material and high soiling conditions make the control of spread and eradication of MRSA without effective disinfection unprofitable. Until now, several examinations with MRSA have been carried out concerning bacteriostasis (Cookson et al., 1991), hand disinfectants (Kampf et al., 1997, Kampf et al., 1998), disinfection of medical instruments (Tekin et al., 2003), antiseptics (McLure and Gordon, 1992, Reimer et al., 2002) and room disinfection applying disinfectant fog (Berrington and Pedler, 1998; Kristoffersen et al., 2006; Kratzer et al., 2006; Clark et al., 2006; Bartels et al., 2008). The results of these studies are probably useful for disinfections in the fields of human medicine and in small animal veterinary practices, but do not answer the differing requirements of disinfection in larger animal houses. Consequently, we tried to determine the efficacy of two chemical disinfectants against a representative strain of MRSA under standardized laboratory conditions in suspension and germ carrier tests. Thus, it appears to be likely that the obtained results will be also applicable under field conditions. The first disinfectant contains organic acids, the second disinfectant aldehydes. Both of these substance classes are widely used in veterinary practice and animal production. They evaporate easily under room temperatures. It can, therefore, be expected that these compounds will work also efficacious in gaseous forms.

We tested both products in a concentration of 1% as recommended by DVG for preventive disinfection (column 4b) and specific disinfection (column 4a) of bacteria (except of bacterial spores) (Anonymous, 2007b). For explanation, the DVG enters products in the list if they have been tested according to the guidelines of DVG in

TABLE 1: *Results of examinations for determination of minimal bacteriostatic concentrations without and with inactivation substances in two independent tests (I and II)*

Test strain	Disinfectant	Test No	cfu[1]/ml (in working culture)	Minimal bacteriostatic concentration (vol. %)						
				Inactivation substance[2]						
				without[3]	1	2	3	4	5	6
S. aureus (Methicillin-resistant)	Venno® Vet 1 super	I	5.3 x 10^8	0.016	0.008	0.008	0.25	0.25	0.25	n. d.
		II	3.6 x 10^8	0.016	n. d.	n. d.	0.25	0.125	0.25	n. d.
	M&enno® Veterinär B neu	I	5.3 x 10^8	0,125	n. d.	n. d.	0.063	0.125	0.125	n. d.
		II	3.6 x 10^8	0,125	n. d.	n. d.	0.063	0.063	0.125	n. d.
	Control (Phenol)	I	5.3 x 10^8	0,25	n. d.	n. d.	0.5	0.5	n. d.	0.25
		II	3.6 x 10^8	0,25	n. d.	n. d.	0.5	0.5	n. d.	n. d.

TABLE 2: *Results of qualitative suspension tests without and with protein load at 20°C (I and II = two independent tests)*

Test strain	Disinfectant	Test No	cfu[1]/ml (in working culture)	Reaction time (min)							
				without protein				with protein			
				5	15	30	60	5	15	30	60
S. aureus (methicillin resistant)	Venno® Vet 1 super (1%)	I	6.2 x 10^8	-	-	-	-	-	-	-	-
		II	3.6 x 10^8	-	-	-	-	-	-	-	-
	M&enno® Veterinär B neu (1%)	I	6.2 x 10^8	-	-	-	-	-	-	-	-
		II	3.6 x 10^8	-	-	-	-	-	-	-	-
	Control 1 (hard water)	I	6.2 x 10^8	+	+	+	+	+	+	+	+
		II	3.6 x 10^8	+	+	+	+	+	+	+	+
	Control 2 (1% phenol)	I	6.2 x 10^8	+	+	-	-	+	+	+	-
		II	3.6 x 10^8	+	+	-	-	+	+	+	-

TABLE 3: *Results of carrier tests at 20°C*

Test strain	Disinfectant	Test No	cfu[1]/ml (in working culture)	Reaction time (min)		
				30	60	120
S. aureus (methicillin resistant)	Venno® Vet 1 super (1%)	I	6.2 x 10^8	-	-	-
		II	3.6 x 10^8	-	-	-
	M&enno® Veterinär B neu (1%)	I	6.2 x 10^8	-	-	-
		II	3.6 x 10^8	-	-	-
	Control 1 (hard water)	I	6.2 x 10^8	+	+	+
		II	3.6 x 10^8	+	+	+
	Control 2 (3% formalin)	I	6.2 x 10^8	-	-	-
		II	3.6 x 10^8	-	-	-

[1] cfu: colony forming unit.
[2] for formula of inactivation substances see material.
[3] Tripticase Soy Broth without inactivation substance.
n. d: not done.
- : free of replicating bacteria.
+ : replicating bacteria present.

suspension and carrier tests by two accredited experts and determined as effective against four test strains (*Staphylococcus aureus*, ATCC 6538, DSM 799; *Enterococcus faecium*, DSM 2918; *Proteus mirabilis*, ATCC 14153, DSM 788; *Pseudomonas aeruginosa*, ATCC 15442, DSM 939). The results of carrier tests are decisive for the registration in the column 4a, and results of suspension tests for column 4b. Our results show that the tested MRSA field strain can be completely disinfected using a concentration of 1% within 5 min in suspension and within 30 min on and in wood carriers. On the basis of the obtained results it can be argued that the application recommendations of DVG for both disinfectants (preventive disinfection: 1%/0,5 h, specific disinfection: 1%/2 h) ensure disinfection of MRSA at 20°C also.

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