The Thai Journal of Veterinary Medicine

Volume 40
Issue 4 *December, 2010*

Article 9

12-1-2010

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Original Article

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Abstract

The antimicrobial activities of organic releasing chlorine disinfectant (Virusnip™) was evaluated and compared by semi-quantitative suspension-neutralization method under clean condition *in vitro*. The test organisms were clinical bacterial isolates of hemolytic *Escherichia coli*, *Salmonella* Typhimurium, *Salmonella* Enteritidis, *Salmonella* Hadar, *Salmonella* Infantis, and *Salmonella* Virchow. Fungal isolates were *Candida albicans* and *Trichophyton mentagophytes*. Four sets of Virusnip™ concentrations were prepared at 1:100, 1:200, 1:400 and 1:1000 for bacteria and 3 sets of 1:100, 1:200 and 1:400 for fungi. Each set of various concentrations was kept at room temperature for 1, 2 and 7 days before use. The assays were performed at room temperature and evaluated after 30 sec, 2 and 60 min contact time between bacterial suspension and Virusnip™ solution, and after 5, 30 and 60 min contact time between fungal suspension and Virusnip™ solution. The antimicrobial activities were calculated in percentage of reduction in viability of the test organisms. The result indicated that Virusnip™ solution kept for 7 days exhibited antibacterial activity at 1:1000 concentration by 100% killing of *E. coli* within 2 min. Comparison of the antibacterial activity among *Salmonella* isolates after 2 min contact of all ages of Virusnip™ solution showed that *S.* Typhimurium and *S.* Hadar were completely destroyed at the lowest 1:400 concentration. Additionally, *S.* Infantis and *S.* Virchow except *S.* Enteritidis were destroyed at the lowest 1:200 concentration. At 1:1000 solution, some serovars required at least 60 min to be killed. The antifungal activity indicated that at least 1:200 of all ages solutions could destroy 100% *T. mentagophytes* within 5 min exposure. The highest dilution 1:400 of all ages of solution could completely reduce both two fungal isolates within 30 min.

Keywords: Antimicrobial activities, bacterial isolates, fungal isolates, Virusnip™

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บทคัดย่อ

การทดสอบประสิทธิภาพ *in vitro* ของสารอินทรีย์ที่หลั่งคลอรีน (Virusnip™) ในการทำลายเชื้อ *Escherichia coli*, *Salmonella* spp. *Candida albicans* และ *Trichophyton mentagophytes*

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การทดสอบประสิทธิภาพในห้องปฏิบัติการของสารฆ่าเชื้อที่เป็นสารอินทรีย์ที่หลั่งคลอรีน หรือ Virusnip™ กระทำโดยวิธี Semi-quantitative suspension-neutralization จุลินทรีย์ที่ใช้ในการทดลอง คือ เชื้อแบคทีเรียชนิด Hemolytic *Escherichia coli*, *Salmonella* Typhimurinum, *Salmonella* Enteritidis, *Salmonella* Hadar, *Salmonella* Infantis, และ *Salmonella* Virchow เชื้อรา ได้แก่ *Candida albicans* และ *Trichophyton mentagophytes* เตรียมสารละลาย Virusnip™ 4 ชุดที่ความเข้มข้น 1:100, 1:200, 1:400 และ 1:1000 สำหรับเชื้อแบคทีเรีย และที่ความเข้มข้น 1:100, 1:200 และ1:400 สำหรับเชื้อรา น้ำยาทดสอบแต่ละชุดถูกเก็บไว้นาน 1, 2 และ 7 วันที่อุณหภูมิห้องก่อน การทดสอบกระทำที่อุณหภูมิห้อง โดยให้สารแขวนลอยของเชื้อแบคทีเรียสัมผัสกับน้ำยาแต่ละชุดเป็นเวลา 30 วินาที 2 นาที และ 60 นาที ในกรณีที่เป็นเชื้อรา ให้สัมผัสกับสารแขวนลอยของเชื้อราเป็นเวลา 5, 30 และ 60 นาที หลังจากนั้นสังเกตการเจริญเติบโตของเชื้อในอาหารเลี้ยงเชื้อชนิดน้ำที่เหมาะสม คำนวณประสิทธิภาพของน้ำยาจากอัตราร้อยละของการลดจำนวนเชื้อจุลินทรีย์ที่มีชีวิต ผลการทดลองพบว่าน้ำยา Virusnip™ ที่เก็บไว้ 7 วันความเข้มข้น 1:1000 สามารถลดจำนวนเชื้อ *E. coli* ได้ 100% ภายในเวลา 2 นาที เมื่อเปรียบเทียบผลการทำลายเชื้อ *Salmonella* ภายใน 2 นาทีของน้ำยาที่เก็บไว้ทุกอายุ ความเข้มข้นต่ำที่สุด 1:400 พบว่า *S.* Typhimurium และ *S.* Hadar ถูกทำลายได้หมด ส่วนความเข้มข้นที่ทำลาย *S.* Infantis และ *S.* Virchow ได้หมดยกเว้น *S.* Enteritidis ที่น้อยที่สุดคือ 1:200 ถ้าความเข้มข้น 1:1000 จะใช้เวลาอย่างน้อย 60 นาทีจึงทำลายเชื้อ *Salmonella* ได้หมดบางซีโรวาร์ สำหรับเชื้อรา พบว่า น้ำยาที่เก็บไว้ทุกอายุ ความเข้มข้นน้อยที่สุด 1:200 สามารถลดจำนวนเชื้อ *T. mentagophytes* ได้ 100% ภายใน 5 นาที ถ้าเจือจางที่ 1:400 สามารถลดจำนวนเชื้อราทั้งสองได้ 100% ภายใน 30 นาที

คำสำคัญ: การทำลายเชื้อ เชื้อแบคทีเรีย เชื้อรา Virusnip™

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Introduction

The modern domestic animal farm environment with high animal density provides an appropriate medium for pathogen replication contribute to high disease incidence especially in poultry and pig industries (Ruano et al., 2001). Disinfectants and antiseptics are important factors of the required levels of hygiene on farms and food-processing premises. However, farm buildings, equipment and tools, rough surfaces, high organic soiling, low temperature and other involved circumstances are strongly limiting factors in this matter. Regular cleaning and disinfection together might be the appropriate way for reduction of microorganisms such as viruses, bacteria and fungi on the involved surfaces to minimize the incidence of diseases transmitted from animal to animal and animal to man. Salmonellosis and Colibacillosis are known to be important zoonotic diseases that cause economic losses in pig and poultry industries worldwide (Acha et al., 2001; Jacob et al., 2005; Fairbrother, 2006; Kabir, 2010). *C. albicans*, the opportunistic pathogen, has been identified in bedding, feed and water supplies of pig. It is implicated in chronic gastroenteritis in piglets, gastric ulceration, cutaneous and oropharyngeal infection (Taylor, 2006), also, associated with comb, lesion of mouth and crop, and stunted growth in poultry (Nagamura et al., 1997; Osorio et al., 2007). Contaminated egg shells may be the source of infection in incubator at hatching time (Gordon and Jordan, 1982). Additionally, *T. mentagophytes* is one of the significant causal agents of human ringworm in many areas of the world (Cabañes, 2000). Animal species including pig and poultry serve as reservoirs and its infection has been considerable zoonotic importance (Gordon and Jordan, 1982). Yeast form of *C. albicans* and fungal spores of *T. mentagophytes* can

persist in the environment. Although those fungi do not cause big loss of livestock industries, both of them are considered as test fungi in evaluation of disinfectants (Terleckyj and Axler, 1987).

The choice of disinfectants is critical in establishing a successful sanitation programme, as not all disinfectants are effective against major pathogens that cause economic diseases in the livestock industry. Virusnip™ an advanced soluble powder disinfectant, is recommended for cleaning and disinfecting all types of farm operation to reduce the risk of herd losses due to diseases and therefore increases profitability. It contains two active ingredients which are potassium monopersulphate (PMP) as oxidizing agent and sodium dichloroisocyanurate (SDIC) as organic releasing chlorine. The antimicrobial action of Virusnip™ derived from organic source of chlorine (SDIC), which provides a higher concentration of available chlorine and are less susceptible to inactivation by organic matter than sodium hypochlorite (McDonnell and Russell, 1999). In water, SDIC generates hypochlorites ready to disinfect instantly, destroy the cellular activity of proteins and inhibit DNA synthesis of microorganisms (McDonnell and Russell, 1999). Once the microorganisms are destroyed, they form a halide by-product and are immediately regenerated back into new active SDIC by PMP, providing continuous action until all the monopersulphate is used up (manufacturer's information). The aim of this study was to evaluate the efficacy of Virusnip™ versus *Escherichia coli*, *Salmonella* spp., *C. albicans* and *T. mentagophytes* in terms of contact time, concentration, and age of ready-to-use solution.

Materials and Methods

Organisms: Organisms used in this study to assess the efficacy of Virusnip™ were from animal or environment of animal origin based on their importance in animal and/or human diseases. Hemolytic *E. coli*, *S.* Enteritidis, *S.* Typhimurium, *S.* Hadar, *S.* Virchow, and *C. albicans* were obtained from Department of Microbiology, Faculty of Veterinary Science, Chulalongkorn University. *T. mentagophytes* was from Central Veterinary Laboratory. *S.* Infantis were received from Department of Medical Science, Ministry of Public Health. *E. coli* was identified from biochemistry properties by method of Barrow and Feltham (1993) while *Salmonella* isolates were identified (Barrow and Feltham, 1993) and serotyped according to Kauffmann and White scheme (Popoff and Le minor, 1997). Additionally, *C. albicans* and *T. mentagophytes* were identified as earlier described (Carter, 1984). All isolates were kept at room temperature, bacterial isolates in stock agar and fungal isolates in sabouraud dextrose agar (SDA-Difco).

Biocide: Virusnip™ (Novartis Ltd.) was obtained in soluble powder from the manufacturer. The compound was diluted in sterile tap water, 4 sets of 1:100, 1:200, 1:400 and 1:1000 concentrations in ready-to-use solutions for bacterial experiment and 3 sets of 1:100, 1:200 and 1:400 for fungal experiments. Each Virusnip™ set test solution was kept at room temperature for 1, 2 and 7 days respectively before use. The contact times of each concentration were 30 sec, 2 and 60 min against each bacterial isolates, and 5, 30 and 60 min against each fungal isolates.

Test methods:

Bacterial preparation: Hemolytic *E. coli* and *Salmonella* isolates from stock agar were subcultured 1 or 2 times in tryptic soy agar (TSA-Difco) incubated at 37^{o}C for 18-20 hrs. The inoculum suspension preparation was performed in overnight broth cultures of brain heart infusion (BHI-Difco) and enumerated in duplicate by standard microbiological procedures to make 108 CFU/ml of each isolate on TSA. The bacterial suspension was freshly prepared for each set of experiment of 1, 2, and 7 day old Virusnip™ solution.

Conidial suspension preparation: Stock of conidial suspensions of *T. mentagophytes* was prepared from 7 day culture in SDA and modified from European Standard (1997) cited by Tortarano et al. (2005). Briefly, macroconidia was detached from culture surface using a glass spatula and transferred into 10 ml sterile distilled water containing 0.05% (w/v) tween 80 in a flask containing glass beads to disperse hyphae from macroconidia. After shaking for 1 min, the suspension was filtered through a gauze, then centrifuged at 2000 rpm for 20 min. The macroconidia were washed two times by resuspension and centrifugation and the number was adjusted to 10^{5}-10^{6} cell/ml in haemocytometer. The viable count was confirmed by standard microbiological method on SDA. The conidial suspension was kept at 4^{o}C for use within 2 weeks. The same procedure was performed with *C. albicans* except that there were not any beads in the flask.

In vitro efficacy testing: The antibacterial activity of Virusnip™ to all bacterial isolates was determined by semi-quantitative suspension-neutralization method under clean condition based on German Society for Hygiene and Microbiology (DGHM) guideline (Reybrouck, 2004). Briefly, one milliliter of the bacterial suspension in overnight broth culture was inoculated into each 9 ml of 1:100, 1:200, 1:400 and 1:1000 Virusnip™ concentrations to obtain approximately 10^{7} CFU/ml in each set of solution. Antibacterial activities were determined for the contact time of 30 sec, 2 and 60 min at room temperature. At the end of each specified contact time, 0.03 ml (one drop) test mixture was added to each set of five replicates 10 ml Dey/Engley neutralizing broth (a combination of 0.5% pancreatic digest of casein, 0.25% yeast extract, 1% dextrose, 0.1% sodium thioglycollate, 0.6% sodium thiosulfate, 0.25% sodium bisulfite, 0.5% polysorbate80, 0.7% lecithin, and 0.002% bromcresol purple-D/E-Difco), incubated at 37^{o}C for 48 hrs. The result was assessed 'growth' by color change of the medium in the tubes from purple to yellow and 'no growth' by showing the original purple. An unclear color change tube was confirmed by subculture one loopful of suspicious D/E broth onto TSA, incubated at 37^{o}C for 48 hrs for colonies growth.

The antifungal activity against conidia of *T.*

mentagophyte and yeast form of *C. albicans* were performed and modified from the method of Tortarano et al. (2005). The procedure was initially examined in the suspension test as mentioned above, the working dilution contained approximately 10^4-10^5 cell/ml, the difference was the contact times of 5, 30 and 60 min at room temperature. At the end of each contact time, 1 ml test mixture was inoculated into 8 ml D/E neutralizing and 1 ml of distilled water. After a neutralization time of 5 min, 0.03 ml neutralizing mixture was transferred to each five replicates 10 ml sabouraud dextrose broth (1% Enzymatic digest of casein+4% dextrose-SDB-Difco) compared with 0.03 ml dropped into five replicates of 6 ml SDA slants. Tubes were incubated at room temperature and observed daily for 21 days. The result was determined 'growth' detected by turbidity in SDB compared with the appearance of the fungal colonies on SDA. The tube was assessed 'no growth' with clear SCB confirmed by no colony on SDA. The antimicrobial activities were calculated in percentage of reduction in viability of microorganisms from the number of 'no growth' tubes in five replicates broth culture based on the interpretation of the capacity test of Kelsey and Sykes' method (Kelsey and Sykes, 1969).

Control tests: The first control was uninoculated D/E broth. The second control was that the 0.03 ml Virusnip™ solution was transferred into one D/E broth tube test for disinfectant contamination. The third control was the effect of Virusnip™ residue in the inoculums performed by transferring 0.03 ml of various concentrations of different ages Virusnip™ solutions and 1 ml of stock bacterial/conidial suspension into 9 ml of each of the replicates broth cultures. D/E broth for bacteria was incubated at 37°C for 48 hrs. SDB for fungi was incubated at room temperature for 21 days. The fourth control was the effect of tap water with pH 6.7 to the test organisms. It was determined by transferring 1 ml of stock bacterial/conidial suspension into 9 ml sterile tap water, mixed together, and left for 60 min, then 0.03 ml was inoculated into each of the five replicates. The results that showed no effects of both Virusnip™ residue and tap water to the test experiments determined by cell growth in both five replicates of D/E and SDB broth cultures.

Results

Bacterial test experiment is shown in Table1. Virusnip™ solution kept as long as 7 days exhibited antibacterial activity at the lowest 1:1000 concentration by completely destroying (100%) hemolytic *E. coli* within 2 min. Comparison of the antibacterial activities among *Salmonella* isolates of 1, 2 and 7 days old solution at 1:100 to 1:400 concentrations, by 2 min contact time, showed that *S.* Typhimurium and *S.* Hadar were completely destroyed while *S.* Enteritidis was destroyed only by the 1 day-old solution. The dilutions for *S.* Infantis and *S.* Virshow to be completely killed were 1:100 and 1:200. At 1:1000 concentration, these Salmonella isolates required at least 60 min to be reduced.

All given concentrations of 1 day-old Virusnip™ solution could completely destroy *T. mentagophytes* within 5 min. However, the antifungal activities at 1:100 and 1:200 solution kept longer for 2 and 7 days still showed the same figure of 100% reduction of *T. mentagophytes* growth by 5 min contact, less extent for *C. albicans*, which were affected only by the 1 and 2 day-old solutions. At 1:400 solution aged 2 and 7 days, 100% cell reduction of both *C. albicans* and *T. mentagophytes* at 30 min exposure were observed (Table2). The number of SDB 'growth' or 'no growth' tubes corresponded to the same number of agar slant tubes. Appearance of fungal colonies on SDA was interpreted as survival of fungus of the timed exposure to the disinfectant.

Table 1. Antibacterial activity of Virusnip™ solution against bacterial isolates

Organism	Time stored of Virusnip™ solution (day)	% Reduction											
		1:100			1:200			1:400			1:1000		
		30 sec	2 min	60min	30 sec	2 min	60min	30 sec	2 min	60min	30 sec	2 min	60min
Haemolytic *E. coli*	1	100	100	100	100	100	100	100	100	100	100	100	100
	2	100	100	100	100	100	100	100	100	100	0	100	100
	7	100	100	100	100	100	100	100	100	100	0	100	100
S. Typhimurium	1	100	100	100	100	100	100	100	100	100	0	0	100
	2	100	100	100	100	100	100	100	100	100	0	0	100
	7	100	100	100	100	100	100	100	100	100	0	0	100
S. Enteritidis	1	100	100	100	100	100	100	100	100	100	0	0	100
	2	0	0	100	0	0	100	0	0	100	0	0	100
	7	0	0	100	0	0	100	0	0	100	0	0	100
S. Hadar	1	100	100	100	80	100	100	60	100	100	0	0	100
	2	100	100	100	100	100	100	60	100	100	0	40	60
	7	100	100	100	60	100	100	60	100	100	0	40	60
S. Infantis	1	100	100	100	100	100	100	80	100	100	0	0	100
	2	100	100	100	100	100	100	80	80	100	0	0	100
	7	100	100	100	100	100	100	60	80	100	0	0	0
*S.*Virchow	1	100	100	100	100	100	100	0	0	100	0	0	80
	2	100	100	100	100	100	100	0	0	100	0	0	0
	7	100	100	100	100	100	100	0	0	80	0	0	0

Table 2 Antifungal activity of Virusnip™ solution against fungal isolates

Organism	Time stored of Virusnip™ solution (day)	% Reduction								
		1:100			1:200			1:400		
		5 min	30 min	60 min	5 min	30 min	60 min	5 min	30 min	60 min
Candida albicans	1	100	100	100	100	100	100	0	100	100
	2	100	100	100	100	100	100	0	100	100
	7	100	100	100	0	100	100	0	100	100
Trichophyton mentagophytes	1	100	100	100	100	100	100	100	100	100
	2	100	100	100	100	100	100	0	100	100
	7	100	100	100	100	100	100	0	100	100

Discussion

Antimicrobial activity of Virusnip™ is based on the function of product, concentration and contact time. From our study, the semi-quantitative way by inoculating in multiple culture broths was used to simplify the evaluation as previous studies (Saitanu, 1985; Davitiyananda et al., 1996), modified from the qualitative suspension test of German Society for Hygiene and Microbiology (DGHM) (Reybrouck, 2004). Neutralizing broth was used for neutralizing the toxicity of the biocide and recovery microorganisms. It is important for the accuracy of a biocidal assay as microbicidal activity is commonly measured as survivors with time. Inhibition of microbial growth by low level of residual biocide would mislead microbicidal activity. D/E broth, which comprises of nutrients required for the replication of microorganisms and substances inactivating a variety of disinfectants and antiseptic chemicals, was chosen as neutralizer in the experiment. Neutralizing component that inactivated the concentration of chlorine in 10 ml of medium final dilution is sodium thiosulphate (Terleckyj and Axler, 1987; Buck and Rosenthal, 1996). The efficacy of neutralization from D/E broth against most biocides for most of the organisms such as *C. albicans*, *Aspergillus fumigatus* (Sutton et al., 1991), *E. coli*, *Salmonella* Cholerasuis, *Staphylococcus aureus* and *T. mentagophytes* etc. was validated (Sutton et al, (2002).

The used concentrations were obtained from the application rates of the manufacturer as follow; 1:100 to 1:200 for destroying viruses, bacteria and fungi on farm buildings, equipments and hatcheries and 1:200 to 1:1000 for drinking water systems. As such, it is noteworthy that Virusnip™ (1 day-old solution) achieved in destroying 100% both bacterial and fungal isolates at the recommendation (1:100 and 1:200) dilutions in vitro. The same concentrations also completely killed classical swine fever virus, porcine reproductive and respiratory syndrome virus, and pseudorabies virus by 30 sec to 5 min (Bunpapong et al., 2010). Furthermore, all isolates could be 100% destroyed at different times exposure and concentrations even the solution was 7 days old, indicating the long lasting action of the product. The duration of microorganisms contacted with the tested Virusnip™ was designed corresponding to the routine application such as short time of footbathing, disinfecting vehicle passing track etc. and the long contact time for other surfaces cleaning. Under farm condition, the tested dilutions are suggested to disinfect after appropriate cleaning.

Virusnip™ appeared to show the best antibacterial effect against hemolytic *E. coli* than *Salmonella* isolates. Kumar et al. (2007) also found that E. coli O157:H7 was significantly more sensitive to stabilized oxychloro (SOC)-based sanitizer compared with Salmonella applied to decontaminate seeds destined for sprout protection. One explanation was an intracellular, *Salmonella*, pathogen that has evolved protective mechanisms to avoid being inactivated by the oxidative stress incurred during interaction with macrophages (Sly et al., 2002). Therefore, it is plausible that *Salmonella* could resist the early oxidative stress response from contact to SOC or Virusnip™ in our study, but subsequently was inactivated through continuous exposure (Kumar et al., 2007). The differences in susceptibility of *Salmonella* serovars in our experiment may reflect from previous repeated exposure to sublethal concentrations of commonly used farm disinfectants of each serovar resulting in different adaptive resistance such as *S.* Enteritidis was more resistant than other isolates. Explanation from report of Randall et al. (2007) stated that 3 from 5 strains of *S.* Typhimurium mutants recovered after one exposure to either disinfectant (tar oil phenol, an oxidizing compound based, an aldehyde based) and required longer exposure time to disinfectants than parent strains to generate a 5 log kill. Generally, molds are more resistant than yeasts. The development of retaining longer time exposure of *C. albicans* to disinfectant may presumably by mutation (Hugo and Russel, 1998). Efficacy of Virusnip™ was supported by other reports that found SDIC already alone to be more effective than sodium hypochlorite (chlorine) against many agents e.g. *Vibrio cholerae* (Eiroa and Porto, 1995), molds, yeasts, total coliforms, *E. coli* and *Salmonella* spp. at the concentration of 200 ppm (Nacimento et al., 2003) except higher concentration of available chlorine and less susceptible to inactivation by organic matter than sodium hypochlorite (McDonnell and Russell, 1999).

In conclusion, Virusnip™ accomplished in completely destroying both bacterial and fungal isolates at the recommended dilutions *in vitro*. The antimicrobial activity of ready-to-use solution can last long at least 7 days at different contact times and concentrations against each isolate. These data offers a scientific basis of antimicrobial properties in suspension of new generation disinfectant which is recommended for use in a wide range of animal farms. The proper concentration can allow the disinfectant to be effective on diverse applications to minimize the risk of disease transmission and provide good health of animals.

Acknowledgement

The authors thank Novartis Animal Health, Switzerland for the financial support in the current study.

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