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Bactericidal Efficacy of a Disinfectant Composed of Povidone-iodine Against *Clostridium Perfringens* and *Mycobacterium Fortuitum*

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ABSTRACT - *Clostridium perfringens* (*C. perfringens*) and *Mycobacterium fortuitum* (*M. fortuitum*) are associated with considerable diseases in animals and human. In this study, the disinfection efficacy of a commercial disinfectant composed to povidone-iodine (PVI) was evaluated against *C. perfringens* and *M. fortuitum*. A bactericidal efficacy test by broth dilution method was used to determine the lowest effective dilution of the disinfectant following exposure to *C. perfringens* and *M. fortuitum* for 30 min at 4°C. The disinfectant and test bacteria were diluted with hard water (HW) or organic matter suspension (OM) according to treatment condition. On HW condition, the bactericidal activity of the disinfectant against *C. perfringens* and *M. fortuitum* was 50 and 80 fold dilutions, respectively. On OM condition, the bactericidal activity of the disinfectant against both *C. perfringens* and *M. fortuitum* was 15 fold dilutions. As the disinfectant composed to PVI possesses bactericidal efficacy against *C. perfringens* and *M. fortuitum*, the disinfectant solution can be used to control the spread of bacterial diseases.

Key words : Povidine-iodine, *Clostridium perfringens*, *Mycobacterium fortuitum*, Disinfectant efficacy

Clostridium perfringens (*C. perfringens*) is a Gram-positive, rod-shaped, anaerobic, spore-forming bacterium of the genus *Clostridium*[1]. The bacterium is ever present in nature and can be found as a normal component of decaying vegetation, marine sediment, and the intestinal tract of humans and other vertebrates[2].

C. perfringens is one of the most frequently isolated bacterial pathogens in foodborne disease outbreaks in humans[3,4]. *C. perfringens* is reported to be the third most common cause of foodborne illness in the world[5] and annually ranks as one of the most common causes of food poisoning in the industrialized world[6-8]. Different meats, including poultry meat, have frequently been reported as the most common food vehicles[5,9]. The common occurrence of *C. perfringens* in raw meat and retail foods may be due to the contamination of carcasses and meat with the intestinal contents of the animals during the slaughtering process[10].

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Mycobacterium fortuitum (*M. fortuitum*) is a rapidly growing mycobacterium and broadly presents world-wide in various habitats such as lakes, surface water, potable water and soil[11]. In addition, *M. fortuitum* has been detected in the environment of pig farms and unpasteurized milk[12]. Furthermore, *M. fortuitum* causes nosocomial infections in human throughout contaminated water sources in hospitals[13]. *M. fortuitum* isolations are not uncommon in normal or diseased domestic and pet animals. The organism has been isolated from skin and diseased organs and also from fluids and excretions of dogs, cats, horses, cattle, pigs and nearby birds and fish including salmonids[12,14].

Epidemiological evidence suggests that *C. perfringens* enterotoxin (CPE) plays an important role in the pathogenesis of both food-borne and non-food-borne human gastrointestinal (GI) illnesses caused by *cpe*-positive *C. perfringens* type A isolates[15]. Specifically, feces from patients suffering from GI illnesses caused by *cpe*-positive isolates contain CPE at concentrations that cause GI effects in animal models[16]. A previous study demonstrated that *M. fortuitum* induced strong apoptosis in human acute monocytic leukemia cells (THP-1) and murine bone marrow-derived macrophages and dendritic cells[17]. In murine infection

model, *M. fortuitum* did not invade recombination activating genes (RAG) murine kidney cell line, while the bacilli infected and proliferated freely inside murine macrophages[18].

In the food industry, thermal treatments typically applied during meat processing are sufficient to kill *C. perfringens* vegetative cells. However, its spores may survive and become highly committed to germinate, outgrow, and multiply to hazardous levels[19,20]. These heat-activated spores are a major concern to food processors, especially during improper chilling after cooking or stored under abusive temperature, conditions that allow a rapid proliferation of *C. perfringens*[21]. In bovine milk, the presence of *Mycobacterium* spp. including *M. fortuitum* has emerged as a public-health concern, especially among individuals who consume raw milk and related dairy products[22].

The wide use of antimicrobial agents in raises an important question regarding the emergence and spread of antimicrobial resistance primarily in the resident normal enteric flora[23]. Antibiotic-resistance of *C. perfringens* and *M. fortuitum* isolates has been reported in several countries[24,25].

As antibiotic-resistant strains of *C. perfringens* and *M. fortuitum* have been increasing due to abuse and overuse of antibiotics, the effective cleaning and disinfection regimes are essential for the prevention of infections and outbreaks[26,27]. As not all disinfectant are effective against the major pathogens including *C. perfringens* and *M. fortuitum*, the choice of disinfectants is critical in establishing a successful sanitation program. Biocides are often composed of a mixture of ingredients that act upon a wide range of cellular mechanisms and targets, which makes it difficult for bacteria to become resistant to biocides[28].

Several disinfectants including chlorine dioxide, betaine hydrochloride and propylene glycol have been used for decontamination of farmed animal and food borne diseases[29,30]. However, there is not the efficacy test for the disinfectant composed of povidone-iodine (PVI) against *C. perfringens* and *M. fortuitum*. Therefore, this study was carried out to examine bactericidal efficacy of a disinfectant solution composed to PVI against *C. perfringens* and *M. fortuitum*.

Materials and methods

Bacteria and culture

C. perfringens (KVCC-BA1100001) and M. fortuitum (ATCC 6841) were obtained from the Korean Veterinary Culture Collection (KVCC, Anyang, Korea) and Rural Development Administration (RDA)-Genebank Information Center (Jeonju, Korea), respectively. The strains were maintained as frozen glycerol stock. *C. perfringens* and *M. fortuitum* were cultured in cooked meat medium (BD Korea Co., Ltd., Incheon, Korea) and Middlebrook 7H9 broth (BD-Korea, Seoul, Korea), respectively, and incubated at 37°C under 5% CO_2 condition for 24 h and 7 days, correspondingly. After incubation, the number of bacteria in culture was measured using the McFarland method[31].

Disinfectant

The active ingredient for the tested disinfectant solution, Betadine® Solution-Concentrate, is PVI 10 g per 100 mL. The disinfectant was provided by Korea Pharma Co., Ltd. (Seoul, Korea). The disinfectant solution was stored in the dark in room temperature and prepared for dilution on the day of evaluation. Determination of the bactericidal efficacy of the disinfectant was based on Animal and Plant Quarantine Agency (Anyang, Korea), Regulation No. 2013-34.

Diluents and treatment condition

Testing was based on bactericidal effects of disinfectant diluents in two treatment conditions (standard hard water (HW) condition and organic matter (OM) condition) and pathogen control (disinfectant negative control) in Table 1. HW, an ingredient of HW treatment condition, was made by adding anhydrous $CaCl_2$ 0.305 g and $MgCl_2 \cdot 6H_2O$ 0.139 g into one liter distilled water. Organic suspension, an ingredient of OM treatment condition, is a solution of 5% (w/v) yeast extract in HW. The test organism was prepared by titration of cultural broth into at least 10^8 colony forming unit (CFU)/mL viable organisms with the same kind of diluents of treatment condition.

Experimental procedures

For the efficacy test against *C. perfringens,* the disinfectant was diluted 40, 45, 50, 55, 60 and 65 times with HW, and diluted 12.0, 13.5, 15.0, 16.5, 18.0 and 19.5 times with OM, respectively. And the disinfectant for the test against *M. fortuitum* was diluted 64, 72, 80, 88, 96 and 104 times with HW, and diluted 12.0, 13.5, 15.0, 16.5, 18.0 and 19.5 times with OM, respectively.

To verify the lowest effective dilution of the disinfectant, five serial dilutions of the disinfectant were prepared and

Table 1. Experimental design for the determination of the bactericidal efficacy of the disinfectant composed to PVI

Treatment condition*	Contents according to treatment condition†			
	HW	OM	Disinfectant	Bacteria
HW condition	+	-	+	+
OM condition	+	+	+	+
Bacteria control	+	-	-	+

*HW: standard hard water, OM: organic matter.
†+: presence, -: absence

placed at 4°C prior to test reaction. 2.5 ml of each disinfectant dilution was mixed with the same amount of test organism followed by contact time of 30 min at 4°C. During this period, the mixture was shaken at 10 min interval. At the end of 30 min contact period, one mL of the mixture was neutralized with 9 mL of Nutrient broth containing 5% inactivated horse serum (BD Korea Co., Ltd., Incheon, Korea) at 37°C. 0.1 mL of the neutralized reaction mixture of *C. perfringens* and *M. fortuitum* was subcultured into 10 mL of recovery each cultural broth at 37°C, 5% CO_2 for 24 h and 7 days, respectively, in incubator. For the test of each bacteria control, 2.5 ml of hard water was mixed with the same amount of each test organism followed by contact time of 30 min at 4°C, and then all procedure were undertaken in parallel for the disinfection test.

The valid dilution of the disinfectant was determined that the greatest dilution showing no growth in four or more in the five replicates was confirmed. The final dilution time was statistically determined by a median value among three valid dilution of the triplicate test, but each value of which should be within 20% experimental error. In each bacteria control, the number of bacterial growth in the five replicates was counted.

Results

Table 2 and 3 shows the final valid dilution of the disinfectant composed to PVI. When the bactericidal effect on HW condition was evaluated, the antibacterial activity of the disinfectant against *C. perfringens* and *M. fortuitum* showed on 50 and 80 fold dilutions, respectively. With the investigation of the bactericidal effect of the disinfectant on OM condition, both *C. perfringens* and M. fortuitum were inactivated on 15 fold dilutions. Because the organic material interferes with efficacy by either inactivating the disinfectant or blocking it from surface contact, the bactericidal activity of the disinfectant on the OM condition was lowered against pathogenic bacteria compared with HW conditions. In the bacterial control, the growth of *C. perfringens* and *M. fortuitum* was verified in all replicates.

Table 2. Final valid dilution of the disinfectant composed to PVI against *C. perfringens*

Bacterial strains	Treatment condition[1)]										
	HW				OM				BC		
	DT	1	2	3	DT	1	2	3	1	2	3
C. perfringens	40	×	×	×	12.0	×	×	×	+[‡]	+	+
	45	×	×	×	13.5	×	×	×	+	+	+
	50	×	×	×	15.0	×	×	×	+	+	+
	55	o[2)]	×[3)]	o	16.5	o	o	×	+	+	+
	60	o	o	o	18.0	o	o	o	+	+	+
	65	o	o	o	19.5	o	o	o	+	+	+
	Valid		50		Valid		15.0			+	

[1)] HW: standard hard water; OM: organic matter; BC: bacterial control; DT: dilution time.
[2)] o: growth, ×: growth inhibition.
[3)] +: all growth in each replicate.

Table 3. Final valid dilution of the disinfectant composed to PVI against *M. fortuitum*

Bacterial strains	Treatment condition[1)]										
	HW				OM				BC		
	DT	1	2	3	DT	1	2	3	1	2	3
M.fortuitum	64	×	×	×	12.0	×	×	×	+[‡]	+	+
	72	×	×	×	13.5	×	×	×	+	+	+
	80	×	×	×	15.0	×	o	×	+	+	+
	88	o[2)]	×[3)]	o	16.5	o	o	o	+	+	+
	96	o	o	o	18.0	o	o	o	+	+	+
	104	o	o	o	19.5	×	×	×	+	+	+
	Valid		80		Valid		15.0			+	

[1)] HW: standard hard water; OM: organic matter; BC: bacterial control; DT: dilution time.
[2)] o: growth, ×: growth inhibition.
[3)] +: all growth in each replicate.

Discussion

The disinfectant composed to PVI is a potential anti-bacterial disinfectant. PVI is a stable chemical complex of polyvinylpyrrolidone (povidone, PVP) and elemental iodine[32]. In addition, PVI is a highly efficient broad-spectrum germicidal agent and effective against bacteria, viruses, fungi, and protozoa[33]. It is widely-used for topical cleansing and wound treatment. PVI releases free iodine, which has an important role in the bactericidal effect of PVI solution through the oxidizing effects of released iodine on proteins and fatty acids[34]. Similarly, through the cytotoxic effects of free iodine, PVI is also an effective tumoricidal agent that may be used as an irrigation fluid to eradicate free cancer cells during head, neck, and colorectal cancer surgery[35-37]. In addition, in contrast to various antibiotic substances which act on the cell walls, PVI not only destroys bacteria, but also effectively inhibits the release of pathogenic factors such as exotoxins, endotoxins and tissue-destroying enzymes[38].

Although the slow release of iodine from the PVI complex in solution minimizes iodine toxicity towards mammalian cells, the iodine is delivered to the bacterial cell surface where it penetrates the cell membrane and inactivates key cytosolic proteins, fatty acids, and nucleotides[39]. In the previous study[40], the oral toxicity LD_{50} of PVI for both rats and mice was 10 g/kg body weight, and PVI containing available iodine 6 g/L was non-irritating to rabbit skin and eye.

To determine a practical minimal disinfecting concentration for 10% PVI over different contact times and temperatures, Heiner et al.[41] exposed *Escherchia coli* to various dilutions of 10% PVI for 5, 15, and 30 min at 10, 20, and 30ºC, neutralized with 0.5% sodium thiosulfate, and determined mean viable colony forming units (CFUs). In results, no CFUs were observed after exposure to the 1:100 dilutions and after 15 min of exposure to the 1:1,000 dilutions across experimental temperatures. In addition, Udompijitkul et al.[42] carried out the sporicidal efficacy of disinfectants used in food-processing facilities and domestic kitckens against different *C. perfringens* strains on stainless steel surfaces. Therefore, treatment with 25 ppm idophore sanitizer (1.6% iodine) for 10 min was achieved 2.7 log *C. perfringens* spore reduction, which was the highest reduction than other disinfectants composited to quaternary ammonium compounds or hydrogen peroxide. Phillips and von Reyn[43] reported that PVI at the concentration of 50-150 ppm as free iodine is known for their superior disinfectant activity against *Mycobacterium* spp. including *M. fortuitum*.

With the consideration of previous studies, the disinfectant composed to PVI is a more effective and safe disinfectant than quaternary ammonium compounds and hydrogen peroxide against *C. perfringens* and *M. fortuitum*.

In the present study, the disinfectant efficacy of the disinfectant composed to PVI has limitation that the results are based on *in vitro* test. Organic material in suspension (OM condition) could not represent all possible parameters of *C. perfringens* and *M. fortuitum* contaminated environment.

As the efficacy of the disinfectant composed to PVI against *C. perfringens* and M. fortuitum was investigated *in vitro*, a controlled field trial is required to determine whether use of the disinfectant will be able to reduce new pathogenic bacteria infection in animal farm and food industry area.

요 약

Clostridium perfringens (*C. perfringens*)와 *Mycobacterium fortuitum* (*M. fortuitum*)은 동물과 사람에서 심각한 질병과 관련이 있는 세균들로 알려져 있다. 본 연구에서는, povidone-iodine을 주성분으로 하는 소독제의 살균효과를 *C. perfringes*와 *M. fortuitum*을 대상으로 평가하였다. 소독제의 살균효과는 배지희석법을 이용하여, 대상 세균들을 4ºC에서 소독제에 30분 동안 노출시킨 다음, 가장 낮은 소독제의 살균 희석배수를 결정하였다. 소독제는 경수와 유기물로 희석하였으며, 경수 조건에서, *C. perfringes*와 *M. fortuitum*에 대해 효과적인 소독제 희석배수는 각각 50과 80배이었다. 유기물 조건에서는, *C. perfringes*와 *M. fortuitum*에 대해 효과적인 소독제 희석배수는 모두 15배로 나타났다. 이상의 결과로 부터, povidone-iodine을 주성분으로 하는 소독제는 *C. perfringes*와 *M. fortuitum*에 대해 살균효과를 갖는 것으로 확인되었으며, *C. perfringes*와 *M. fortuitum*에 의한 질병의 확산을 방지하기 위해 사용될 수 있을 것으로 사료된다.

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