DETECTION OF *LEGIONELLA* SPECIES IN WATER SAMPLES FROM SURANAREE ARMY HOSPITAL

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การตรวจหาแบคทีเรีย *Legionella* ในตัวอย่างน้ำจากโรงพยาบาลค่ายสุรนารี

นางสาวอารียา กลิ่นโพธิ์กลาง

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต

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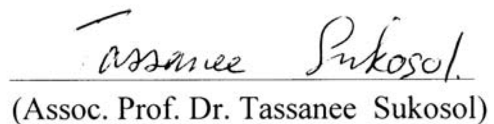

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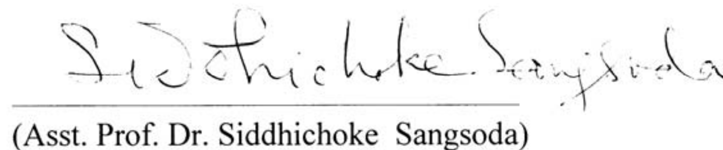

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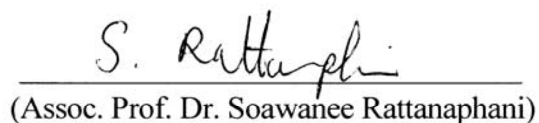

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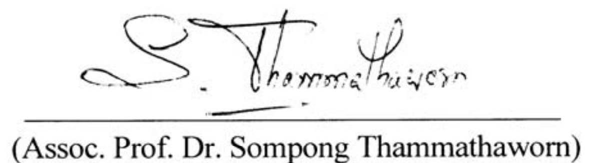

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Legionella โดยเฉพาะ *Legionella pneumophila* เป็นจุลชีพฉวยโอกาสที่ก่อให้เกิดโรคปอดอักเสบลีเจียนแนร์ (Legionnaires'disease) ในผู้ที่มีภูมิต้านทานโรคต่ำ เช่น คนไข้ที่รักษาตัวในโรงพยาบาล และพบเชื้อได้ทั่วไปในแหล่งน้ำต่างๆ การศึกษานี้มีวัตถุประสงค์เพื่อตรวจหา *Legionella* ในตัวอย่างน้ำจากโรงพยาบาลค่ายสุรนารี จังหวัดนครราชสีมา เพื่อประโยชน์ในการเฝ้าระวังการระบาดของโรคลีเจียนแนร์ การเพาะเชื้อจากตัวอย่างน้ำประปาและตัวอย่าง biofilm ซึ่งเก็บจากฝักบัว ก๊อกน้ำและระบบทันตกรรม จำนวน 109 ตัวอย่าง พบ *L. pneumophila* 4 ตัวอย่าง (3.67%) ผลดังกล่าวไม่ต่างจากผลการศึกษาอื่นๆที่ทำในประเทศไทยซึ่งพบประมาณ 2.6-6% เชื้อ *Staphylococcus*, *Pseudomonas*, *Acinetobacter*, *Alcaligenes* และ *Flavobacterium* พบ 9.17, 89.0, 76.15, 57.8 และ 49.54% ตามลำดับ และตรวจไม่พบแบคทีเรียกลุ่มโคลิฟอร์ม สำหรับน้ำในระบบทันตกรรม 9 ตัวอย่างจากชุดทำฟัน 3 ชุด ทุกตัวอย่างตรวจไม่พบ *Legionella* แต่พบแบคทีเรียรวมมากกว่ามาตรฐานที่สมาคมทันตแพทย์อเมริกากำหนดไว้ (ไม่เกิน 200 CFU ต่อมิลลิลิตร) หลังจากการกำจัดเชื้อในแหล่งตัวอย่างที่พบ *Legionella* แล้ว ได้ทำการเพาะเชื้อซ้ำอีกครั้ง ไม่พบ *Legionella* และแบคทีเรียอื่นๆ ยกเว้น *Pseudomonas* และ *Acinetobacter* ในการทดลองได้มีการเปรียบเทียบผลของ pre-treatment ด้วยกรด ความร้อน และความร้อนร่วมกับ saponin 1% ต่อการเจริญของ *L. pneumophila* ATCC 33152 พบว่าผลของ pre-treatment ทั้ง 3 วิธี ไม่มีความแตกต่างอย่างมีนัยสำคัญ ($p<0.05$) และ saponin ไม่มีผลต่อจำนวน *L. pneumophila* ในตัวอย่าง biofilm เมื่อนำ *L. pneumophila* ATCC 33152 มาทดสอบความไวต่อคลอรีน พบว่าไวต่อคลอรีนที่ระดับความเข้มข้น 0.1-0.5 มิลลิกรัมต่อลิตร ภายหลังการสัมผัสเชื้อ 3 ชั่วโมง นอกจากนี้ในตัวอย่างน้ำทั้งหมดตรวจไม่พบคลอรีนอิสระ (0 มิลลิกรัมต่อลิตร) ในขณะที่การประปานครหลวงแห่งประเทศไทยได้กำหนดมาตรฐานของคลอรีนอิสระไว้ที่ 0.2-0.5 มิลลิกรัมต่อลิตร

สาขาวิชาจุลชีววิทยา ลายมือชื่อนักศึกษา อารียา กลิ่นโพธิ์กลาง

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AREEYA KLINPHOKLANG : DETECTION OF *LEGIONELLA* SPECIES IN WATER SAMPLES FROM SURANAREE ARMY HOSPITAL. THESIS ADVISOR : ASSOC. PROF. TASSANEE SUKOSOL, Ph.D. 86 PP. ISBN 974-533-562-2

LEGIONELLA/WATER SAMPLE/HOSPITAL

Legionella especially *L. pneumophila* are recognized as opportunistic pathogen causing nosocomial Legionnaires'disease in immuno-compromised person such as patients in the hospitals. They were found in worldwide aquatic environments. This study aims to detect *Legionella* from water samples in Suranaree Army Hospital, Nakhon Ratchasima province for the vigilance of the outbreak of Legionnaires'disease. A total of 109 municipal tap water samples and biofilm samples from showerheads, faucets and dental units were cultured. Four samples (3.67%) were positive for *L. pneumophila*. This result was likely the same as other studies in Thailand that was 2.6-6%. *Staphylococcus*, *Pseudomonas*, *Acinetobacter*, *Alcaligenes* and *Flavobacterium* were found 9.17, 89.0, 76.15, 57.8 and 49.54%, respectively. All samples were not found coliform bacteria. From dental unit water systems, all 9 samples from 3 dental unit sites were not found *Legionella* but carried more viable microorganisms than the maximum allowable load recommended by American Dental Association (≤200 CFU/ml). After decontamination at the *Legionella* positive sites, samples from decontaminated sites were repeated cultivation. *Legionella* and other microorganisms were not found, except *Pseudomonas* and *Acinetobacter*. In this study, the comparison of the effects of 3 pre-treatments (heat, acid and heat with 1%

saponin) which affected the growth of *L. pneumophila* ATCC 33152 showed non significantly difference ($p<0.05$). The effect of saponin on *L. pneumophila* biofilm samples showed no observable difference in the microbial quantity. The susceptibility of *L. pneumophila* ATCC 33152 to free chlorine was observed at 0.1-0.5 mg/l after 3 hours of the exposure. The free chlorine concentrations of all collected water samples were not detectable (0 mg/l) whereas the Metropolitan Waterworks Authority, Thailand recommended the standard level of free chlorine at 0.2-0.5 mg/l.

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Academic Year 2005 Advisor's Signature Tassanee Sukosol

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LIST OF ABBREVIATIONS

$	Dollar (dollars)
%	percent
ADA	American Dental Associations
BCYE	Buffered Charcoal Yeast Extract Agar
°C	Degree celsius
CFU/ml	Colony Forming Units per millilitre
CFU/swab	Colony Forming Units per swab
DFA	Direct fluorescence antibody
DNA	Deoxyribonucleic acid
DPD	*N,N*-diethyl-*p*-phenylenediamine
e.g.	exempli gratia
et al.	et alia (and other)
(m, μ) g	(milli, micro) Gram
(μ) g/l	(micro) Gram per litre
GVPC	Glycine Vancomycin Polymyxin B and Cycloheximide Agar
h	Hour
i.e.	id est
(m, μ)l	(milli, micro) Litre
M	Molar
mmol	Millimole

LIST OF ABBREVIATIONS (Continued)

ml/l	Millilitre per litre
min	Minute
MWA	Metropolitan Waterworks Authority, Thailand
N	Normal
PCR	Polymerase chain reaction
sp., spp.	Species (singular, plural)
rRNA	Ribosomal ribonucleic acid
wt/vol	Weight by volume

CHAPTER I

INTRODUCTION

1.1 Significance of the study

Legionellae and specifically *Legionella pneumophila* were first recognized during the highly publicized outbreak of Legionnaires' disease, which occurred among people who had attended an American Legion Convention in Philadelphia in 1976. Among 221 persons who contacted the disease, 34 subsequently died (Maiwald *et al.*, 1998). Legionellae are now recognized as opportunistic pathogens of human and an important cause of pneumonia (Fliermans, 1996). From a microbiological standpoint, the legionellae are most notable for their complex ecology. *Legionella* is the largest genus of bacteria that can survive, almost exclusively, as parasite of protozoa (Kwaik *et al.*, 1998). Legionellae are primarily associated with aquatic environments, although they have been isolated from potting soils and moist soil samples (Fliermans, 1996). They have a worldwide distribution but until recently, they appear to play a limited role as human pathogens (Fields, 1996). Industrial setting and larger populations of immunocompromised persons have led to increase the infections in humans (Sabria and Yu, 2002). Legionnaires' disease is usually a consequence of altering the environment for human benefit. The overall attack rate of pneumonia in the United States is 8,000-18,000 cases each year (Fields *et al.*, 2002; Pasculle, 2000). Pneumonia is the sixth leading cause of death in the United States,

with an estimated annual cost of $23 billion. *L. pneumophila* causes 4.1-20.1% of community-acquired cases, many of which result in hospitalization (Waterer *et al.*, 2001). These data suggest that *L. pneumophila* is the major cause of serious cases of pneumonia.

The investigation of a number of epidemic and sporadic cases has shown that *L. pneumophila* is a common cause of both community-acquired and nosocomial pneumonia (Steinert *et al.*, 2002). Nosocomial Legionnaires' disease has become increasingly common, contributing up to 30% of hospital-acquired pneumonia in some institutions (Sabria and Yu, 2002). In the earlier reports of nosocomial legionellosis, mortality rates were as high as 80%, usually in immunosuppressed patients who did not receive appropriate antibiotics. However, mortality in the USA has decreased from 46% in 1982 to 14% in 1998 with increased awareness and increasing empirical use of quinolones for hospital-acquired pneumonia (Sabria and Yu, 2002). In reality, this disease is a common form of severe pneumonia, but these infections are infrequently diagnosed because legionellae are fastidious and not easily detected. Failure to diagnose Legionnaires' disease is largely due to a lack of clinical awareness. Routine environmental culture of the hospital water supply for legionellae has proven to be an important strategy in prevention of nosocomial legionellosis (Sabria and Yu, 2002).

In Thailand, since 1984-2002, there were 16 patients, have been reported to be infected with Legionnaires' disease. Fifteen patients were infected by *L. pneumophila* and another one was infected by *L. jordanis* (สมชัย บวรกิตติ, 2546; Srisawai *et al.*, 1984). *Legionella* spp. have been isolated from man-made and environmental samples

in several regions of Thailand (ทิพวรรณ กังแฮ และเกสร บุญยรักษ์โยธิน, 2547; สมชัย บวรกิตติ, 2546; วันทนา ปวีณกิตติพร และคณะ, 2547; Tishyadhigama *et al.*, 1995). Moreover, European Working Group for *Legionella* infection and Communicable Disease Surveillance had reported that 11 travelers who checked in Thai hotels in several provinces such as Bangkok, Suratthanee, Krabi, Chiang Mai and Chonburi (Pattaya), got Legionaires' disease and 3 of them died (สมศักดิ์ ชัยพิพัฒน์ และคณะ, 2543). This report affected tourism in Thailand, therefore, the public health authorities should be aware of the possible opportunistic infection of this disease. Also routine environmental culture of water samples is necessary to monitor the outbreak of legionellosis.

The purpose of this thesis is to study the prevalence of legionellae and tentative pathogenic microorganisms in hospital water samples and this result will make the hospital staffs to beware the outbreak of nosocomial legionellosis.

1.2 Research objectives

1) To study the prevalence of *Legionella* spp. in Suranaree Army Hospital water samples and the positive sites will be reported to the hospital, after water treatment to get rid of the microorganisms, water samples at those sites will be cultured again to examine that the tentative pathogenic microorganisms are left or not.

2) To determine the relationship between water parameters (such as temperature, pH and free chlorine content) and the prevalence of legionellae.

3) To detect the other microorganisms which will indicate the quality of water samples.

1.3 Research hypothesis

Legionellae are found in aquatic environments worldwide, so they may be found in water samples from Suranaree Army Hospital, Nakhon Ratchasima Province.

1.4 Scope and limitations of the study

At least 50 water samples (such as hot-water from shower heads and faucets) will be collected from Suranaree Army Hospital in Nakhon Ratchasima Province. The water samples will be determined for *Legionella* spp., total heterotrophic plate count, gram-negative bacteria, *Staphylococcus* spp. and total coliforms by cultural technique. The positive sample sites of *Legionella* spp. will be repeatedly examined after they have been decontaminated.

1.5 Expected results

The data of the prevalence of *Legionella* spp. from water samples in Suranaree Army Hospital will be achieved. The relationship between water parameters and the prevalence of *Legionellla* spp. including the microbiological quality of water samples will also be obtained. The information of this study will be used to monitor the outbreak of nosocomial legionellosis.

CHAPTER II

LITERATURE REVIEW

2.1 *Legionella* species

According to Bergey (1984), *Legionella* are rod shaped, 0.3-0.9 µm in width and 2-20 µm or more in length, do not form endospores or microcysts, not encapsulated, non acid-fast, gram-negative, motile by one, two or more straight or curved polar or lateral flagella but nonmotile strain are occasionally seen. They are aerobic, L-cysteine-HCl and iron salts are required for their growth. Most species produce beta-lactamase and liquefy gelatin. The oxidase reaction is variable, and reactions for nitrate reduction, urease and carbohydrate utilization are negative. Amino acids are the carbon source for legionellae. Strains belonging to all serogroups of *L. pneumophila* except serogroups 4 and 15 strongly hydrolyze hippurate. All legionellae contain large amounts of branched-chain cellular fatty acids and contain ubiquinones with side chains of 9-14 isoprene units (Fields *et al.*, 2002).

The number of species and serogroups of legionellae continue to increase. As previously mentioned, there are currently 48 species comprising 70 distinct serogroups in the genus *Legionella* (Table 1). There are 15 serogroups of *L. pneumophila* and two each in *L. bozemanii*, *L. longbeachae*, *L. feeleii*, *L. hackeliae*, *L. sainthelensi*, *L. erythr*a and *L. quinlivanii*, and a single serogroup in each of the remaining species. A single species of *Legionella*, *L. pneumophila*, caused approximately 90% of all documented cases by *L. pneumophila* serogroup 1 (Ruef,

1998). Approximately one third of the 48 species of *Legionella* have been associated with human diseases under appropriate conditions.

Table 1 *Legionella* species and serogroups

Species	No. of serogroups	No. associated with disease
1. *L. pneumophila*	15	15
2. *L. bozemanii*	2	2
3. *L. dumoffii*	1	1
4. *L. micdadei*	1	1
5. *L. longbeachae*	2	2
6. *L. jordanis*	1	1
7. *L. wadsworthii*	1	1
8. *L. hackeliae*	2	2
9. *L. feeleii*	2	2
10. *L. maceachernii*	1	1
11. *L. birminghamernsis*	1	1
12. *L. cincinnatiensis*	1	1
13. *L. gormanii*	1	1
14. *L. sainthelensi*	2	2
15. *L. tucsonensis*	1	1
16. *L. anisa*	1	1
17. *L. lansingensis*	1	1
18. *L. erythra*	2	1

Table 1 (continue)

Species	No. of serogroups	No. associated with disease
19. *L. parisiensis*	1	1
20. *L. oakkridgensis*	1	1
21. *L. spiritensis*	1	0
22. *L. jamestowniensis*	1	0
23. *L. santicrucis*	1	0
24. *L. cherrii*	1	0
25. *L. steigerwaltii*	1	0
26. *L. rubrilucens*	1	0
27. *L. israelensis*	1	0
28. *L. quinlivanii*	2	0
29. *L. brunensis*	1	0
30. *L. moravica*	1	0
31. *L. gratiana*	1	0
32. *L. adelaidensis*	1	0
33. *L. fairfieldensis*	1	0
34. *L. shakespearei*	1	0
35. *L. waltersii*	1	0
36. *L. genomospecies*	1	0
37. *L. quateirensis*	1	0
38. *L. worsleiensis*	1	0
39. *L. geestiana*	1	0

Table 1 (continue)

Species	No. of serogroups	No. associated with disease
40. *L. natarum*	1	0
41. *L. londoniensis*	1	0
42. *L. taurinensis*	1	0
43. *L. lytica*	1	0
44. *L. drozanskii*	1	0
45. *L. rowbothamii*	1	0
46. *L. fallonii*	1	0
47. *L. gresilensis*	1	0
48. *L. beliardensis*	1	0

Source: Fields *et al.* (2002).

2.2 Legionellosis

The diseases caused by *Legionella* are collectively termed legionellosis. The two principal diseases are Legionnaires' disease (*Legionella* pneumonia) and Pontiac fever (Garnett *et al.*, 1990). Legionnaires' disease is an often fatal pneumonic illness (Percival *et al.*, 2000). It has an incubation period of 2-10 days and is predominantly caused by *L. pneumophila* (Hurst and Knudsen, 1997). Early symptoms of the disease include malaise, myalgias, anorexia, headache and muscle aches. It rapidly progresses to high fever (>40°C), unproductive cough and shortness of breath (Garnett *et al.*, 1990; Pasculle, 2000; Stout and Yu, 1997). Although no chest x-ray pattern can separate this infection from other types of pneumonia, alveolar infiltrates are more common with Legionnaires' disease (Fields *et al.*, 2002; Pasculle, 2000).

Extrapulmonary systems, such as diarrhea, neurological abnormalities, renal involvement and relative bradycardia are frequent (Maiwald *et al.*, 1998). Hyponatremia (serum sodium concentration, ≤130 mmol per liter) occurs more frequently in Legionnaires' disease than other types of pneumonia (Stout and Yu, 1997). The attack rate for Legionnaires' disease is low and mainly affects the susceptible people in the community. The host susceptibility is a key factor for developing the illness. The mortality rate of this disease is estimated to be between 5-30% and possibly higher in susceptible persons (Cloud *et al.*, 2000).

Another non-pneumonic form of legionellosis is Pontiac fever. Pontiac fever is a self-limiting, non-fatal, non-pneumonic, febrile, influenza-like illness (Pasculle, 2000). An incubation period is 1-2 days and the attack rate is more than 90% (Maiwald *et al.*, 1998). The first recognized cases of Pontiac fever were caused by *L. pneumophila*, however, a similar illness has been caused by *L. feeleii, L. anisa* and *L. micdadei*. Patients exposed to the same environmental source may develop either Pontiac fever or Legionnaires' disease (Pasculle, 2000).

Pontiac fever patients with seroconvert to *Legionella*, however the microbe has never been isolated. Therefore, it has been speculated that Pontiac fever is caused by viable but non-culturable (VBNC) forms of *Legionella*. Other hypotheses to explain Pontiac fever include changes in virulence factors, toxic or hypersensitivity reactions (Steinert *et al*., 2002).

2.3 *Legionella* ecology

Microbial ecologists recognize that the vigor of aquatic microorganisms general follows the thermal cycle of their habitat. This means that a seasonal change

reflected in the activity and subsequent density of the microbial population in the habitat. *Legionella* follows such a pattern (Fliermans, 1996). Because *Legionella* survives and multiplies in aquatic habitats, many of the bacterium studies have been concerned with the locations and conditions in which the bacteria flourish and the host risks pose by those occurrences.

Legionellae are distributed worldwide and are ubiquitous in aquatic environments. They are naturally found to be low concentration in fresh, brackish and coastal waters and have also been isolated from water-soil environments such as mud and sediment (Fliermans, 1996). The presence of the organisms in these natural aquatic environments is not concerned, as these sources have not been linked with disease in human. Worldwide outbreaks of *Legionella* have been exclusively associated with man-made built environments such as decorative fountains, shower faucets, whirlpool spas and cooling towers (Breiman, 1993). In these environments, the steady liquid state is disturbed due to the operation of the water systems and can produce aerosols that are known to be the primary mode of transmission in causing legionellosis (Fliermans, 1996; Pasculle, 2000).

In the natural habitat, the survival of legionellae will be influenced by other parameters, such as temperature, pH and changes in nutrient availability. These bacteria are capable to survive in the extreme range of environmental conditions (Atlas, 1999; Fliermans *et al*., 1981). Legionellae are heat tolerance and remaining viable when test at temperature between 7-70°C (Bentham *et al*., 1993). They multiply at temperature between 20-45°C, with optimal growth occuring between 35-37°C. In cool environments (<20°C) legionellae will remain dormant and will multiply when temperature rises. They can survive in the hot environments (>50°C),

at 50°C, the survival reduces to a few hours and at 60°C, they survive for only a few minutes (Bentham *et al*., 1993; Garnett *et al*., 1990; Wadowsky *et al*., 1985).

Legionellae can withstand in the wide range of pH from acidic condition (pH 2.0) to basic condition (pH 10.0). In natural water, legionellae have been isolated in the pH range 5.4-8.1 (Wadowsky *et al*., 1985) and the laboratory study has shown that the optimal pH for growth is 6.9-7.0 (Bentham *et al*., 1993; Bergey, 1984).

The stringent requirements for laboratory culture would indicate that legionellae may not be free-living aquatic bacteria outside the laboratory, but may depend on an association with other organisms. Sediment, sludge, scale and organic matter can act as sources of nutrients (Steinert *et al*., 2002). Slime or biofilms, which often forms on the surface of water, provides favorable condition for growth. Nutrients, such as trace elements and amino acids are essential for their growth. Growth may be stimulated by the presence of small amounts of mineral including zinc, iron, magnesium, manganese, potassium, copper and phosphate (Bentham *et al*., 1993; James *et al*., 1999). Naturally occurring legionellae may meet their carbon and energy requirements by using amino acids produced by other organisms which share habitats with them.

2.4 Amplification factors

In natural water sources, legionellae are generally present in very low concentrations. However under certain conditions, usually within manufactured aquatic environments, the concentration of the bacteria may increase markedly (Lee and West, 1991). The environment persistence of legionellae is aided by their ability to adapt to a variety of different ecological niches, either as intracellular parasites of

protozoa, as free-living members of complex biofilm communities, or as planktonic cells (James *et al.*, 1999; Kwaik *et al.*, 1998).

Protozoa appear to have a critical role in the amplification process for legionellae (Kwaik *et al.*, 1998). Biofilms, ubiquitous within plumbing systems also support the growth of the bacteria (Percival *et al.*, 2000). Certain environmental conditions that are beneficial for *Legionella* proliferation include stagnation, sediment, sludge, scale, corrosion and niches which are commonly seen in cooling tower water systems (Fliermans, 1996).

2.4.1 Protozoa associations

Protozoa are commonly found in aquatic environments and are characterized as unicellular, heterotrophic, motile and have no cell wall. They are generally considered to be polymorphic, whereby they undergo several morphological changes throughout their life cycle. Such changes may include alternating between active trophozoites and dormant cysts (Mairer *et al.*, 2000). Legionellae survive in aquatic and moist soil environments as intracellular parasites of free-living protozoa (Kwaik *et al.*, 1998). These bacteria have been reported to multiply in 13 species of amoeba and two species of ciliated protozoa (Table 2).

Table 2 Protozoa supporting the growth of legionellae.

Category	Organism
Amoeba	*Acanthamoeba castellanii*
	A. polyphaga
	A. palestinensis
	A. royreba
	A. culbertsoni
	Naegleria gruberi
	N. fowleri
	N. lovaniensis
	N. jadini
	Hartmannella vermiformis
	H. cantabrigiensis
	Vahlkampfia jugosa
	Echinamoeba exudans
Ciliated protozoa	*Tetrahymena pyriformis*
	T. vorax

Source: Fields (1993).

Protozoa do not only provide nutrients for the intracellular legionellae, but also represent a shelter when environmental conditions become unfavorable. If conditions become unfavorable for protozoa, that is, food availability is poor or the protozoa are subjected to dry conditions, the protozoa form hard and impervious outer protective shell called cyst (Percival *et al.*, 2000). If legionellae are found within these

cysts, they have excellent protective environments against dry conditions, extremes of temperature, and treatment with biocides (Barbaree *et al.*, 1986). As the bacteria are within the cysts, they can be blown away in the air. They have been established because the cysts are able to survive for nearly 400 days in sterile tap water without additional nutrients (Skaliy *et al.*, 1980). If conditions become suitable for growth, they will change to be amoebal cells and *Legionella* can be released. Therefore, this provides a perfect protective environment within very harsh condition (Percival *et al.*, 2000).

Beyond protection and reactivation from dormancy, *Legionella* may also use protozoa to colonize new habitats. In this regard, inhaled protozoa seem to be a vehicle for effective transmission to humans (Steinert *et al.*, 2002). Several aspects of the interaction between legionellae and protozoa suggest that this is a natural relationship contributing to the multiplication within protozoa and it may enhance the ability of legionellae to infect mammalian cells, promote extracellular survival by inducing a stress-resistant phenotype, which is characterized by altered morphology and envelope composition and increased resistance to antimicrobial agents (James *et al.*, 1999).

2.4.2 Biofilm associations

Biofilms have been defined as cells immobilized at a substratum and frequently embedded in an organic polymer matrix of microbial origin (Percival *et al.*, 2000). There are five recognized stages in the development of biofilms (Percival *et al.*, 2000) as follow:

The first step involves the adsorption of organic molecules onto a surface and is considered to be an important step in conditioning film formation. This step is triggered once a solid surface makes contact with an aqueous phase and is dependent on the physiochemical properties of the surface material.

The second step involves the association of microorganisms with the newly conditioned surface and this is achieved when organisms are transported to the surface by mechanisms such as diffusion and electrostatic forces. It has suggested that bacteria (usually the primary colonizers) will begin to formulate the biofilms in response to certain environmental conditions such as nutrient availability.

The third step involves the adhesion of the microorganisms by extracellular polymeric substances, which anchors the primary colonizers onto the surface. Once this step has occurred the process is irreversible.

The fourth step involves an accumulation of microorganism secretions and the addition of microorganisms from the bathing medium. This creates a multi-organism environment whereby all organisms communicate and co-operate to form a complex community.

The fifth step involves biofilm detachment where a disturbance segments of the biofilms to peel off from the surface of the biofilms. Examples of such disturbance include turbulence, microbial antagonism, localized biofilm lysis, or nutrient and oxygen depletion. Protozoan grazing of biofilms may also cause the detachment of biofilms.

Biofilms have been shown to be a contributory factor in supporting the growth of *Legionella* within aquatic environment. Biofilm matrices are known to

provide shelter and a gradient of nutrient (Schwartz *et al.*, 1998). The complex nutrients available with biofilms have led some researchers to propose that the biofilms support the survival and multiplication of legionellae outside the host cells (Fields *et al.*, 2002).

Investigators have attempted to detect extracellular growth of *L. pneumophila* by using a biofilm reactor and a defined bacterial biofilms grown on nonsupplemented potable water. *L. pneumophila* associated with and persisted in the biofilms with and without *H. vermiformis*. *L. pneumophila* cells did not appear to develop microcolonies, and growth measurement studies indicated that *L. pneumophila* did not multiply within the biofilms in the absence of amoebae. This study suggested that *L. pneumophila* may persist in biofilms in the absence of amoebae, but in the model, the amoeba were required for multiplication of the bacteria (Fields *et al.*, 2002).

The biofilms not only provide a source of nutrients for legionellae but also protect them from the antibiotics and other biocides (Schwartz *et al.*, 1998). The control of biofilm development is considered to be vital to the control of legionellosis.

2.4.3 Algal associations

Legionellae have been shown to have symbiotic relationship with some algae and cyanobacteria, which may involve phosphorous metabolism (Bentham *et al.*, 1993; Fliermans, 1996; Garnett *et al.*, 1990). The bacteria derive organic compounds from algal photosynthetic products and are often found in close association with or attach to the surface of algal cells. Extract from cyanobacterial cultures preserves the viability of *Legionella* in aerosols. Some algae e.g. *Fischerella*

spp. produce slimy matrices, which may afford physical protection for bacteria from desiccation and biocide (Garnett *et al*., 1990). From this evidence, it may be concluded that algae and cyanobacteria may play an important role in the colonization and dispersal of *Legionella* in water systems.

2.5 Dissemination of the bacteria to humans

The primary mode of transmission of legionellosis is inhalation of *Legionella* organisms in aerosolized droplets of respirable size (1-5 µm) (Fliermans, 1996; Pasculle, 2000). Transmission occurs occasionally via other routes, including direct inoculation of surgical wounds with contaminated potable water during placement of surgical dressing and aspiration of contaminated water by persons recovering from head and neck surgeries (Ruef, 1998). There continue to be no evidence for person-to-person transmission (Breiman, 1993).

A number of devices have been implicated as sources of aerosol transmission of legionellae, which are shown in Table 3.

Table 3 Known sources for transmission of legionellae via aerosols

Type of water	Transmitting devices
Potable	Showers, tap water devices and respiratory therapy equipment
Nonpotable	Cooling towers and evaporative condensers, whirlpool spas, decorative fountains, ultrasonic mist machines, humidifiers

Source: Breiman (1993).

Numerous epidemic investigations have demonstrated that cooling towers and evaporative condensers have served as sources of *Legionella* infection (Breiman *et al.*, 1990; Cordes *et al.*, 1980; Dondero *et al.*, 1980; Garbe *et al.*, 1985). Both are heat rejection devices and reservoirs filled with fairly warm recirculating water. The condition within cooling tower reservoirs is ideal for the growth of legionellae. The large surface area of the tower basin, fill, pipework and heat exchanger, variable oxygen tension and sites differing in temperature create extensive and various sites suitable for colonization by a variety of microorganisms (Bentham *et al.*, 1993).

Potable water has long been suspected to be a potential source of *Legionella* infection (Breiman, 1993). Aerosolization of contaminated warm potable water via showerheads or tap water faucets can transmit legionellosis (Arnow *et al.*, 1985; Bollin *et al.*, 1985; Cordes *et al.*, 1981). Also respiratory care equipment has served as source of legionellosis (Arnow *et al.*, 1982). In hospitals with contaminated potable water systems, use tap water to wash jet nebulizers and other equipment used to deliver respiratory care likely represent a substantial risk for patients. Many patients are at high risk for legionellosis because of chronic lung disease and/or steroid use. Respiratory equipment filled or rinsed with tap water may serve as a secondary reservoir for legionellae. Subsequent reattachment of the device to the patient could directly instill *Legionella*-containing respirable droplets into the respiratory tract (Woo *et al.*, 1992). A key point elucidated by Mastro *et al.* (1991) was that devices such as medication nebulizers may retain droplets of water 12 h after rinsing.

It has been suggested that *Legionella* spp. within dental lines may contribute to respiratory illness among dentists and dental staffs. Higher rates of seropositivity for *Legionella* antibodies have been found among dental personnel than among the

general public, suggesting that aerosols generated in dental operatories are the source of exposure to *Legionella* spp. Water-cooled, high-speed handpieces generate stable aerosols that may contain *Legionella* spp. The complex design of dental-chair equipment results in the stagnation of water within the water lines, where bacteria, including *Legionella* spp., can survive within biofilms (Atlas *et al.*, 1995).

Other sources of aerosols that have been shown to transmit disease include whirlpool baths, humidifiers, and decorative fountains (Breiman, 1993).

2.6 Host susceptibility

A key factor in the chain of causation is susceptibility of the host. *Legionella* is frequently characterized as an opportunistic pathogen, and it most frequently attacks individual who has underlying illness or weak immune system (Fliermans, 1996).

While infection by *L. pneumophila* may cause clinical disease in healthy person and increase risk in patients with underlying diseases. Nosocomial outbreaks typically affect patients with any of the following conditions: cigarette smoking (Breiman, 1993; Stout and Yu, 1997), alcoholism (Ruef, 1998), chronic obstructive pulmonary disease, and immunosuppression following organ transplantation, malignancy, treatment with steroids and chronic renal insufficiency (Fliermans, 1996; Ruef, 1998; Sabria and Yu, 2002; Stout and Yu, 1997). The data from the Centers for Disease Control's surveillance system suggested that persons with end-stage renal disease have a 200-fold-greater risk of developing *Legionella* infection than do normal hosts (Breiman, 1993). Patients with acquired immunodeficiency syndrome are greater risk of Legionnaires' disease than the general population, however,

legionellae are rarely detected in studied of pulmonary disease among cohorts of HIV patients. This may be due to prophylaxis of HIV-infected patients with trimethoprim-sulfamethoxazole, an antimicrobial agent against *Legionella* species (Fields *et al.*, 2002; Stout and Yu, 1997). However, the clinical manifestation of lung abscesses, extrapulmonary infections, and bacteremia have been observed to be more severe (Stout and Yu, 1997). While pediatric legionellosis has been reported, the disease is extremely rare except among markedly immunosuppressed children (Breiman, 1993). Multivariate analysis of risk factor data have not been done to determine whether advancing age and male gender are independent risk factors or are associated with a greater likelihood for development of chronic underlying disease (Breiman, 1993). In addition, health care workers with predisposing condition may also be at risk for acquisition of Legionnaires' disease (Ruef, 1998).

2.7 Environmental surveillance of *Legionella* spp. in Thailand

Legionella spp. have been isolated in several regions of Thailand. In 1984, Tanaka and the colleagues had studied the epidemiological survey of Legionnaires' disease from environmental sources in Bangkok and Chantaburi. They found that 18.6% of 70 cooling towers were contaminated with *L. pneumophila* (สมชัย บวรกิตติ, 2546).

Tishyadihigama *et al.* (1995) had surveyed for the contamination of legionellae in man-made built environments and environmental samples in many regions of Thailand. They found 57% of 94 cooling towers and 21.8% of 78 other

environmental sources, were contaminated with legionellae and *L. pneumophila* serogroup 1 was the most prevalent organisms.

Kongkankong *et al*. (2000) investigated the quality of water from the dental units at the Faculty of Dentistry, Khon Kaen University. *L. pneumophila* was detected in 6% of water samples and the number of total bacteria count was higher than the recommendation of the American Dental Association (≤200 CFU/ml).

Kanghae and Bunyaraksyotin (2004) had investigated for *Legionella* spp. in hotel cooling towers in 5 tourist provinces in the southern part of Thailand, which were Trang, Krabi, Phuket, Surat Thani and Songkhla from September 2002 to October 2003. They found that 11 of 37 cooling towers (29.7%) were contaminated with *L. pneumophila* serogroup 1, 3, 5, 7, 8 and other *Legionella* spp.

Lertkhanawanichakul (2004) had investigated *Legionella* spp. from the environment at Walailuk University in Nakornsrithammaraj province. A total of 168 samples were taken from 76 water samples from environmental sources, 30 biofilm samples from sink faucets and showerheads taken by cotton swabs and 62 air samples from environmental resources by the air sampler (microflow 90). From water samples, *Legionella* spp. were isolated 2.6% (2 from 76 water samples). From air samples, *Legionella* spp. were found 3.2% (3 from 62 air samples) and these bacteria were not found in 30 biofilm samples. Although, the detection rate for *Legionella* spp. were low.

Paveenkittiporn *et al*. (2004) had investigated the pathogenic organisms in 18 natural hot spring water in 4 northern provinces of Thailand, which were in Mae Hong Son, Chiang Mai, Chiang Rai and Lampang. The temperatures of water samples

are 55->90 °C. Only water sample from Mae Hong Son province that was found *L. pneumophila* serogroup 6.

2.8 Laboratory diagnosis

2.8.1 Cultural method

Cultural method remains to be the gold standard for the identification of legionellae and probably is the most sensitive means for diagnosis when it is performed early in the course of disease. *L. pneumophila* was first isolated by using Mueller-Hinton agar supplemented with hemoglobin and IsoVitaleX (MH-IH) (Feeley *et al.*, 1978). The essential component in hemoglobin was a soluble form of iron, and L-cysteine is the essential amino acid provided by the IsoVitaleX. These refinements led to the development of Feeley-Gorman agar, which provided better recovery of the organisms from tissue (Feeley *et al.*, 1978). Later, starch was replaced with charcoal to detoxify the medium and the amino acid source was changed to yeast extract, resulting in charcoal yeast extract agar (Feeley *et al.*, 1979). Charcoal yeast extract agar is the base form for most media used to grow legionellae. The medium used for the culture of legionellae has been improved several times, eventually resulting in the medium currently used which are buffered charcoal-yeast extract (BCYE) agar enriched with α-ketoglutarate with and without selective agents added (Edelstein, 1981, 1982). Culture requires the use of selective and nonselective media. These media can be prepared with or without indicator dyes. These dyes impart a color specific for certain species of *Legionella* (Vickers *et al.*, 1981). Although the majority of *Legionella* spp. grow on BCYE agar, some require supplementation with bovine serum albumin to enhance growth (Morrill *et al.*, 1990).

On BCYE, *Legionella* spp. generally produce small, slow growing, circular colonies having a cut-glass or beaten copper appearance with entire edge colonies and usually appear after 2-6 days incubation at 35ºC (Feeley *et al.*, 1978). Under a dissecting microscope, colonies show speckled opalescence and near the edge may appear granular blue/green or pink/purple, giving an opal-like appearance (Bentham *et al.*, 1993).

Presumptive identification of legionellae can be obtained by inoculation of suspect colonies onto BCYE agar and BCYE without L-cysteine. Colonies growing on BCYE but not on BCYE without L-cysteine within three days can be presumed to be *Legionella* species. When cultured on BCYE agar, long wave ultraviolet light irradiation may be used to distinguish *Legionella* species. *L. pneumophila* and other species may show a weak yellowish auto-fluorescence (Feeley *et al.*, 1979). *L. anisa*, *L. bozemanii*, *L. dumoffii*, *L. gormanii*, *L. parisiensis*, *L. steigerwaltii* and *L. tucsonensis* show a bright blue/white auto-fluorescence while *L. rubrilucens* and *L. erythra* show a bright red auto-fluorescence (Bentham *et al.*, 1993; Mietzner and Stout, 2002).

For growth, cultural media require pH of 6.9-7.0 with a high relative humidity. Incubation in 2-5% CO_2 atmosphere may give slightly increased colony numbers when culturing from clinical specimens but it is not necessary with environmental samples (Mietzner and Stout, 2002).

Samples can be pre-treated to reduce numbers of other bacteria and so select for *Legionella*. A selective procedure is required to reduce the number of non-*Legionella* bacteria before culturing some water samples with high total bacterium concentrations. Non-*Legionella* bacteria can be selectively killed by either

acid pre-treatment or brief exposure to higher temperature. Legionellae are more resistant to lower pH and brief exposure to higher temperatures than many other freshwater bacteria. For acid pre-treatment, the sample is mixed and incubated with an acid buffer (KCl-HCl, pH 2.2) for 3-30 min (Bopp *et al.*, 1981). The sample is usually neutralized with a 1.0 N KOH buffer before inoculation onto media. Heat pre-treatment is accomplished by incubating sample at 50°C for 30 min (Verran *et al.*, 1995). These two techniques use the temperature tolerance and short term low pH tolerance of legionellae as selective methods.

2.8.2 Non-cultural methods

Several non-cultural methods have been developed to detect legionellae in environmental samples. These methods offer the potential of greatly increased sensitivity. Culture remains to be the method of choice for detected legionellae, primarily because non-cultural methods cannot provide information regarding the viability of the bacteria. These non-cultural methods include detection of the organisms with specific antisera by direct fluorescence antibody (DFA) staining and procedure to detect nucleic acids of legionellae by polymerase chain reaction (PCR).

Legionella sp. can be detected by DFA staining and this was formerly an important rapid diagnostic test for legionellosis. The number of specific antisera is the limitation of the use of DFA to detect legionellae. Since there are no antisera, which specifically react, with all *Legionella* species, a different antiserum must be used for each species or serogroup. Reports on the sensitivity and specificity of DFA testing of environmental specimens vary greatly, some studies indicating that the test is relatively insensitive and nonspecific. The use of PCR for detecting nucleic acid of

legionellae in the environment has proved to be a valuable technique for the legionellosis investigation. Amplification of DNA by PCR is very sensitive tool, able to detect the DNA equivalent to one *Legionella* specimen (Waterer *et al.*, 2001). PCR is also able to screen for all *Legionella* species and serogroups within a single test. A number of legionella genes, including 5S rRNA, 16S rRNA and the macrophage infectivity potentiator (*mip*) genes have been used as target for PCR (Cloud *et al.*, 2000).

CHAPTER III

MATERIALS AND METHODS

3.1 Chemicals and Reagents

All chemicals and reagents were laboratory grades and analytical grades, and purchased from Carlo Erba Reagenti (Carlo Erba, Italy), Sigma-Aldrich Co. (Sigma, U.S.A.) and Merck KGaA (Merck, Germany).

3.2 Instrumentation

Instruments for the detection of *Legionella* spp. in water samples from Suranaree Army Hospital were located in the Instrument Building of the Center for Scientific and Technology Equipment, Suranaree University of Technology, Nakhon Ratchasima Province, Thailand.

3.3 Samples' collection and processing

Suranaree Army Hospital located in Nakhon Ratchasima Province, Thailand. Total of 59 samples sites were selected for detection of *Legionella* spp. The water sample sites which were showerheads, faucets and dental unit water systems, generated aerosol to possibly exposed patients. The samples were collected both water and biofilm samples from the same sites.

3.3.1 Shower heads and faucets

Water and biofilm samples from showerheads and sink faucets were collected by the modified method of Cordes *et al.* (1981). Showerheads and sink faucets were turned on, and the initial 500 ml of water samples was collected in sterile containers. The biofilms were collected by scraping the internal surface of each detached showerheads or faucets with a sterile cotton swab, the cotton tip was collected in a sterile screw-capped tube with a 10 ml aliquot of water from the same source.

3.3.2 Dental unit water systems (DUWS)

Water samples from DUWS were collected from the oral rinsing cups, the handpieces and the turbines (Walker, *et al.*, 2000; Zanetti, *et al.*, 2000). Approximately 500 ml of water sample was collected in the sterile container. In order to create favorable conditions for *Legionella* spp., the sample was collected in the morning before starting the work, so that the water stagnated in the water line at least 12 h.

3.3.3 Samples' processing

The 0.1-0.5 ml of 0.1 N sodium thiosulfate ($Na_2S_2O_3$) (Appendix B3) was added to each water sample to neutralize the disinfectants (Mietzner and Stout, 2002). The samples were shipped in insulated containers and processed within 24 h in the laboratory.

The 100 ml of water samples were concentrated by filtration through 0.2 μm pore-size cellulose nitrate filters (Whatman, Maidstone, England). After

concentration, the membrane filters were aseptically cut into smaller pieces and placed into sterile, screw-capped containers with 10 ml of original water samples. These were vortexed for 30 seconds to dislodge bacterial cells from the membrane filters (Bartie *et al.*, 2003). The swab samples were vortexed for 10 min to dislodge bacterial cells from cotton tips (Bartie *et al.*, 2003).

3.4 Microbiological analysis

3.4.1 Detection of *Legionella* species

The concentrated samples were treated with selected methods to reduce the number of non-legionellae organisms. Portions of the sample concentrate were treated as below.

1) The untreated samples.

2) Acid pre-treatment, 1 ml of sample aliquot mixed with an equal volume of acid buffer (0.2 M HCl-KCl, pH 2.2) (Appendix B1) for 3 min (Ta *et al.*, 1995). After pre-treatment, the sample was neutralized by 1.0 N KOH (Appendix B2) before cultivation.

3) Heat pre-treatment, 1 ml of the concentrated sample was incubated at 50°C in a water bath for 30 min (Verran *et al.*, 1995) before cultivation.

4) Heat pre-treatment combining with 1% saponin treatment, 1 ml of the concentrated sample and 1% saponin (Sigma, U.S.A.) were incubated at 50°C in a water bath for 30 min.

Pre-treatment samples were isolated for *Legionella* spp. by the spread plate technique, 0.1 ml of each sample was placed in duplicate on Buffered Charcoal

Yeast Extract (BCYE) agar (Apppendix A1) and Glycine Vancomycin Polymixin B Cycloheximide (GVPC) agar (Appendix A2). All plates were incubated in a humidified incubator at 35°C for 7 days. Examine all cultures daily after 48 h incubation for the presence of opaque bacterial colonies that had the ground-glass appearance using dissecting microscope. Colonies, which had the typical *Legionella*-like appearance, were tested for cysteine requirement by sub-culture on corresponding quadrants of BCYE and BCYE without L-cysteine agar plates and incubated in a 35°C incubator for 4 days. *Legionella* spp. grew on BCYE but did not grow on BCYE without L-cysteine. *Legionella pneumophila* serogroup 1 ATCC 33152 was used as positive control and sterile distilled water was used as negative control for cultivation of water samples.

L. pneumophila was identified from other legionellae by hippurate hydrolysis reaction (Fox and Brown, 1989). Suspected colonies of legionellae were placed in 1.5 ml microcentrifuge tubes containing 0.4 ml of 1% sodium hippurate (Appendix B4). The mixtures were incubated at 37°C overnight. A 3.5% solution of ninhydrin (Appendix B5) was added, and the mixtures were returned to 37°C. *L. pneumophila* developed a blue color after 5-15 min and the other legionellae would not change or developed a blue-gray color after 15 min. Only a blue color appearing by 15 min will be considered as positive. The number of typical colonies of *Legionella* spp. and *L. pneumophila* were counted, and reported in colony forming units (CFU)/ml.

3.4.2 Isolation and quantitation of total heterotrophic plate count

To determine the viable counts of other heterotrophic bacteria, a 10-fold dilution series of the concentrated water samples and biofilms samples were prepared. The 0.1 ml of each diluted sample was inoculated on plate count agar (PCA) (Appendix A6) in duplicate then spread using the flamed spreaders. All plates were incubated at 35°C for 24-48 h. The numbers of colonies were counted, and reported in colony forming units (CFU)/ml.

3.4.3 Isolation of Gram-negative bacteria

Samples were cultivated for gram-negative bacteria by spread plate technique. The 0.1 ml of sample was placed on selective and differential medium for gram-negative bacteria, MacConkey agar (Appendix A4), in duplicate then spread using the flamed spreaders. All plates were incubated at 35°C for 24 h. Colonies of gram-negative bacteria were identified by morphology and biochemical tests (Gram-stain, catalase test, oxidase test, motility, OF-glucose, TSI, LIA and citrate). Genus of gram-negative bacteria that found in the samples were reported.

3.4.4 Isolation of *Staphylococcus* spp.

Samples were cultivated for *Staphylococcus* spp. by spread plate technique, on selective and differential medium, Baird-parker agar (Appendix A3). *Staphylococcus* typically formed slate gray to jet-black, smooth, entire colonies. If there were *Staphylococcus aureus*, egg yolk clearing might be observed. Then characterized by morphology and biochemical tests (Gram-stain, catalase test and coagulase test).

3.4.5 Isolation and quantitation of total coliform counts

Samples were concentrated by filtration through 0.45 μm cellulose nitrate membrane filters. The membranes were aseptically removed to sterile absorbent pads, which added m-Endo medium (Appendix A5), and then incubated at 35°C for 24 h. All bacteria that produced pink to dark-red color with metallic sheen within 24 h. on Endo-type medium were considered to be coliform group.

Coliform density was calculated by the following equation.

$$\text{Total coliform colony /100 ml} = \frac{\text{coliform colony counted} \times 100}{\text{ml sample filtered}}$$

3.5 Physical and chemical analysis of samples

The temperature of the water samples were measured in the flowing water from the sample sites by thermometer (Brannan, England). The pH value was performed by pH meter (Mettler Delta 320, Mettler-Toledo LTD, England). Free chlorine residual and total chlorine residual contents of the water samples were measured with an *N*,*N*-diethyl-*p*-phenylenediamine (DPD) colorimetric kit (CN-66, Hach, U.S.A).

3.6 Evaluation of microbiological quality of water samples

The microbiological parameters of water samples were compared with the standard of tap water recommended by Metropolitan Waterworks Authority, Thailand

(based on WHO guideline 1993) (Metropolitan Waterworks Authority, 2004) (Appendix C) as follow.

Bacteriology quality of tap water

Total coliform bacteria	not detectable
E. coli	not detectable

Dental unit water system (DUWS) samples were compared with the standard of American Dental Association (ADA) recommendations for DUWS water quality. ADA has set a standard of $\leq$ 200 CFU/ml for the maximum microbial load delivered by DUWS.

3.7 Susceptibility of *Legionella pneumophila* to chlorine

Legionella susceptibility to chlorine was examined at the concentrations that might be found in public water distribution systems. This study was investigated by the modified methods of Skaliy *et al.* (1980) and Kuchta *et al.* (1983) as follow:

3.7.1 Test organisms

L. pneumophila serogroup 1 (ATCC 33152) was cultured on BCYE agar at 35°C. At 72 h of incubation, bacteria were harvested, washed twice in sterile distilled water and suspended in sterile distilled water by vigorous shaking to break aggregated bacteria. The suspension of washed cells was adjusted turbidimetrically to a concentration of 10^7-10^8 cell/ml.

3.7.2 Disinfectant

A 100 mg/l stock chlorine solution was prepared by dissolving calcium hypochlorite in sterile, distilled, deionized water. Free and total chlorine concentrations were measured at the beginning and at the end of each experiment by the amperometric method (APHA, 1992) to ensure that no unexpected chlorine was appeared in test system. Chlorination of the test system was achieved by adding precalculated volumes of this stock to the chlorine-free tap water. The desired chlorine concentrations were prepared and added immediately to washed cells.

3.7.3 Experimental procedures

The bactericidal action of each concentration was measured by exposing 10^5-10^7 viable cell/ml in 500 ml of calcium hypochlorite solution contained in a 1000 ml Erlenmeyer flask. Immediately after cells were added, the suspension was thoroughly agitated, and 10 ml of designated sample was taken at time zero, and 0.1 ml of a 10% (wt/vol) solution of sodium thiosulfate was added. A 1.0 ml aliquot of the neutralized sample was serially diluted (1:10) with sterile distilled water. The 0.1 ml of three suitable dilutions were cultured in duplicate on BCYE agar at 35°C.

Test suspensions were then incubated at room temperature. The number of survivors was subsequently determined after 3, 6, 24 and 168 h. At each time interval, a 10 ml sample was processed as described above.

The controls consisted of 10^5-10^7 cell/ml in 500 ml of sterile tap water without disinfectant and were exposed to the same conditions as the test suspensions.

The number of survivors was determined in term of the number of *L. pneumophila* colonies developing on BCYE agar after 6-7 days of incubation at

35°C. Plates without colonies did not develop were incubated for 14 days before being discarded.

3.8 Effects of pre-treatment on *Legionella pneumophila* biofilms

The experiments were done to determine the effect of 3 pre-treatment methods which were acid, heat and heat with 1% saponin addition on *L. pneumophila* in the biofilm samples.

3.8.1 Test organisms

L. pneumophila serogroup 1 (ATCC 33152) was prepared as same as test organisms in chlorine test (section 3.7.1).

3.8.2 Biofilm model

The biofilm model was designed as the modified method of Schwartz *et al.* (1998). The 1 l flasks were contained unsterile, dechlorinated tap water by boiling before use and inoculated with a pure culture of *Legionella pneumophila.* Pieces of metal material (2×2 cm) were added into three flasks and incubated at ambient temperature for 14 days, under static condition for biofilm formation. Bacteria in biofilms were released with a series (6×2 min) of low–energy sonications.

3.8.3 Experimental procedure

Biofilm samples which attached on metal material coupons were recovered for *L. pneumophila* by culture method as in section 3.4.1.

3.9 Repeated cultivation after decontamination of *Legionella* spp. at the positive sites

The positive sites of *Legionella* spp. were reported to Suranaree Army Hospital. Decontaminations were done according to the recommendation of Department of Health, Ministry of Public Health. Two weeks after decontamination, the repeated samples were collected and cultured again to prove that the tentative pathogenic microorganisms were destroyed completely.

CHAPTER IV

RESULTS

4.1 Survey and collection of samples

The water sample sites for the detection of *Legionella* spp. from Suranaree Army Hospital were selected from the potential sites that could generate the aerosols which might contain legionellae in droplets to the exposed persons. Total of 59 water samples sites (showerheads, faucets and dental unit water systems) were selected from medicine ward, gastrointestinal (GI) tract microscopy room, emergency room and dental unit. Both water and biofilm samples from the same sites were collected. The sources of samples were shown in Table 4.

Table 4 Source of samples for detection of *Legionella* spp.

Source	Specimen	No. of samples
Faucets	Tap water	35
	Biofilms	35
Showerheads	Tap water	15
	Biofilms	15
Dental unit water systems		
- oral rinsing cup	Water sample	3
- handpieces	Water sample	3
- turbine	Water sample	3
Total		**109**

4.2 Detection of *Legionella* species

Total of 59 water samples and 50 swab samples in the same sites were detected for *Legionella* spp. by spread plate technique on BCYE agar and selective medium, GVPC agar (BCYE agar with glycine, vancomycin, polymixin B and cycloheximide). Colonies of *Legionella* spp. on BCYE and GVPC agar are grayish, slightly convex, circular and entire with a ground glass appearance under dissecting microscopy (Figure 1). Young colonies will have iridescent edges with green, pink, or purple color. Older colonies may have a waxy appearance and be sticky when touch with an inoculating loop. Suspected colonies were selected from Gram-negative, thin bacilli (Figure 2) and examined for cysteine requirement by subcultured on BCYE agar and BCYE agar without L-cysteine. Colonies that were unable to grow on BCYE agar without L-cysteine were considered as *Legionella* spp.

Total of 109 samples were evaluated for legionellae by cultural technique. Four samples (3.67%) were positive for *Legionella* spp. and were identified as *L. pneumophila* by positive result for hippurate hydrolysis reaction. Four positive with *L. pneumophila* samples, 2 were from water samples and 2 were from biofilm samples with one positive site (Faucet 14) that was contaminated by *L. pneumophila* in both water sample and biofilm sample. *L. pneumophila* was detected from showerhead and the faucets but not from of the dental units. The density and hazard level of the contaminated samples at the sampling sites are reported in Table 5.

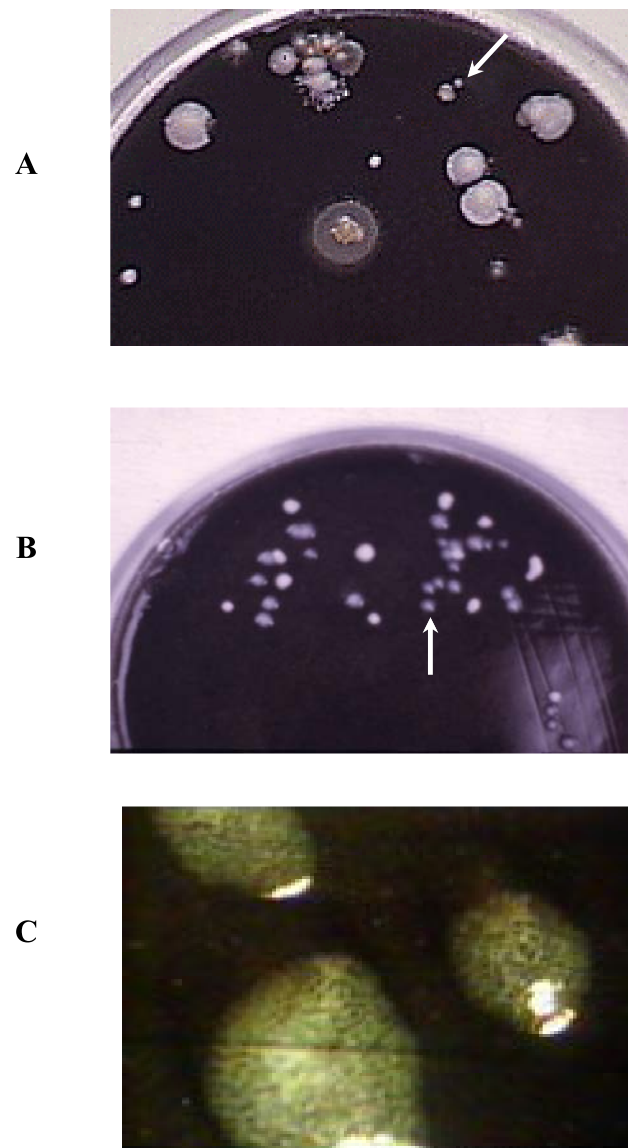

Figure 1 *Legionella* colony (A) grows on GVPC agar for 4 days, the other colonies are contaminated microorganisms. Colonies are grayish, slightly convex and circular (B) with ground glass apperance (C) under dissecting microscope (×10.5). The arrows indicate *Legionella* colonies.

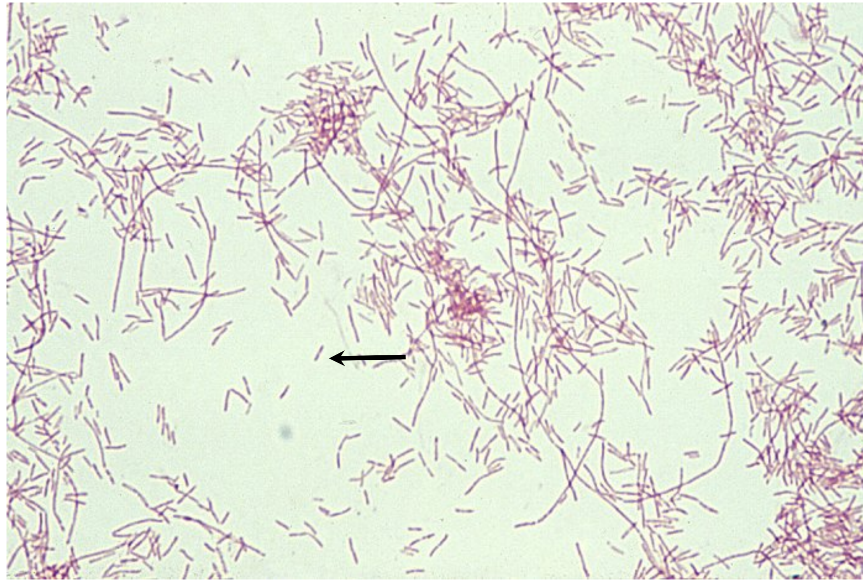

Figure 2 Gram stain of *Legionella* sp., bright field microscopy (x1000). The arrow indicates cell of *Legionella* sp.

The hazard levels were categorized by Shelton and coworkers (1993) (Appendix D). They analyzed 900 samples from building where outbreak of Legionnaires' disease had occurred, building where might be associated with sporadic cases of Legionnaires' disease and building with no case of disease. The numbers of legionellae in water samples were determined and results were categorized by source of samples. The densities of *L. pneumophila* that contaminated in the water samples were low (<1 to 2 CFU/ml) which were indicated in the hazard level 2-3. *L. pneumophila* contaminated in biofilm samples were 80 and 840 CFU/swab which might be indicated in the hazard level 4-5.

Table 5 Density of *Legionella pneumophila* contaminated samples

Sample	Type of sample	Density of *L. pneumophila*	Hazard level[a]
Shower 4	biofilm sample	80 CFU/swab	4 (10-99)
Faucet 14	biofilm sample	840 CFU/swab	5 (≥100)
	water sample	2 CFU/ml	3 (1-9)
Faucet 15	water sample	<1 CFU/ml	2 (detectable, but<1)

[a] Shelton *et al.* (1993)

4.3 Detection of other microorganisms

During this study, 59 water samples and 50 swab samples were collected and cultivated for *Legionella* spp. and other microorganisms (total heterotrophic bacteria, Gram-negative bacteria, *Staphylococcus* spp. and total coliform bacteria). The total viable counts of samples were recorded.

The densities of heterotrophic bacteria that found in each type of samples were shown in Table 6. The average mean of heterotrophic bacteria was 5.00×10^5 and ranged between 0.59×10^2-1.75×10^7 CFU/ml. The densities of heterotrophic bacteria were 1.97×10^4 CFU/ml for water samples and 2.35×10^6 CFU/ml for biofilm samples from showerhead. From faucet samples, there were 5.60×10^3 CFU/ml for water samples and 1.13×10^6 CFU/ml for biofilm samples. The densities of heterotrophic bacteria from dental unit samples were 1.09×10^3, 1.03×10^3 and 1.47×10^7 CFU/ml for turbine, oral rinsing cup and handpieces samples, respectively.

Table 6 Total viable counts (CFU/ml) of samples

Source	Sample no.	Mean	Range (min-max)
Showerhead			
water sample	15	19702	600-166000
biofilm sample	15	2345280	6900-17500000
Faucet			
water samples	35	5597	59-43000
biofilm samples	35	1128235	530-16500000
Dental unit			
turbine	3	1090	340-2400
oral rinsing cup	3	1033	560-1300
handpieces	3	1467	450-2300
Total	**109**	**500343**	**59-17500000**

Moreover, the other microorganisms that indicated the quality of water as indicator organisms (coliform bacteria and *Staphylococcus* sp.) were investigated. The variety of microorganisms in each sample site was shown in Table 7.

Table 7 Other microorganisms in the samples

Sample type	No.	Coliforms	*Staphylococcus* spp.	Gram-negative
Showerheads				
water	15	0/15	0/15	*Pseudomonas, Flavobacterium, Acinetobacter, Alcaligenes*
biofilms	15	ND	3/15	*Pseudomonas, Flavobacterium, Acinetobacter, Alcaligenes*
Faucets				
water	35	0/35	2/35	*Pseudomonas, Flavobacterium, Acinetobacter, Alcaligenes*
biofilms	35	ND	5/35	*Pseudomonas, Flavobacterium, Acinetobacter, Alcaligenes*
Dental unit				
turbine	3	0/3	0/3	*Pseudomonas, Acinetobacter, Alcaligenes*
oral rinsing cup	3	0/3	0/3	*Pseudomonas, Flavobacterium, Acinetobacter*
handpieces	3	0/3	0/3	*Pseudomonas, Flavobacterium, Acinetobacter, Alcaligenes*

ND, Not done

All of water samples had no coliforms contamination but there were *Staphylococcus* sp. 9.17% (10 from 109 samples) and they were found in biofilm samples more than water samples. The Gram-negative bacteria found in the samples

were *Pseudomonas, Acinetobacter, Alcaligenes* and *Flavobacterium*. The distributions of Gram-negative bacteria contamination were shown in Table 8.

Table 8 Gram-negative bacteria isolated from sample sites

Samples	Gram-negative bacteria			
	Pseudomonas	***Acinetobacter***	***Alcaligenes***	***Flavobacterium***
Showerheads				
water	12/15	10/15	7/15	6/15
biofilms	15/15	15/15	10/15	12/15
Faucets				
water	30/35	21/35	14/35	13/35
biofilms	35/35	33/35	30/35	20/35
Dental unit				
turbine	1/3	2/3	1/3	0/3
oral rinsing cup	2/3	1/3	0/3	1/3
handpieces	2/3	1/3	1/3	2/3

From this study, *Pseudomonas* spp. were the highest contaminated Gram-negative bacteria. They were found 89% (97 from 109 samples) and were found in all biofilm samples from showerheads and faucets. *Acinetobacter, Alcaligenes* and *Flavobacterium* were found 76.15%, 57.80% and 49.54%, respectively.

4.4 Physical and chemical analysis of samples

Water samples from Suranaree Army Hospital were examined for pH, temperature and chlorine concentration (total and free chlorine concentration). The results of the water parameters were summarized in Table 9.

Table 9 Physical and chemical values of water samples

Parameters	Mean	Median	Range (min-max)
PH	7.95	7.97	7.69 - 8.27
Temperature (°C)	30.80	32	23 - 37
Chlorine concentration (mg/l)			
Free chlorine	0	0	0-0.1
Total chlorine	0.1	0.1	0.1-0.2

This table showed that the average pH of the water samples was 7.95 and ranged between 7.69 to 8.27. The average temperature was 30.80°C and ranged between 23°C to 37°C. The average chlorine concentrations were 0 and 0.1 mg/l for free chlorine and total chlorine, respectively.

4.5 Microbiological quality of water samples

The microbiological parameters of tap water samples were compared with the standard of tap water recommended by Metropolitan Waterworks Authority, Thailand (based on WHO guideline 1993) (MWA, 2004) (Appendix C). The standard of MWA was suggested that it should not have coliform bacteria and *E. coli* in water systems and also no contaminated legionellae for safety water in hospital.

Dental unit water system (DUWS) samples were cultured and compared with the standard of American Dental Association (ADA) recommendations for DUWS water quality. ADA has set a standard of ≤200 CFU/ml for the maximum microbial load delivered by DUWS.

The results of microbiological quality of tap water samples and dental unit water samples from Suranaree Army Hospital were shown in Table 10.

Table 10 Microbiological quality of water samples from Suranaree Army Hospital

Parameters	**No. of positive/total no.**		**Recommend**
	Tap water	**Dental unit**	
Total coliforms	0/50	0/9	**Not allowable**
E. coli	0/50	0/9	**Not allowable**
Legionella spp.	3/50	0/9	**Not allowable**
Total viable count	-*	9/9**	**≤200 cfu/ml****

*, MWA standard does not recommend this criterion
**, ADA standard for DUWS water quality

All of examined tap water and dental unit water systems were not contaminated with coliform bacteria. It meant that there was no fecal contamination in water systems. Three positive sites with legionellae contamination were not safe and all of nine dental unit water samples had total viable counts exceed than the ADA recommendations.

4.6 Relationships between water parameters and the prevalence of *Legionella pneumophila*

The relationships between *Legionella* recovery and physical, chemical and bacteriological parameters of the samples were examined by linear regression (SPSS 10.0, SPSS inc.). There was no relationship between each parameter and the prevalence of *Legionella pneumophila*. The statistical analysis was shown in Table 11.

Table 11 Statistical analysis of linear regression between water quality parameters and the prevalence of legionellae

Parameters	*r*	*P*
Temperature	-0.121	0.05
PH	-0.116	0.05
Free Cl concentration	-0.020	0.05
Heterotrophic bacteria	-0.050	0.05
Coliforms	-	0.05
Staphylococcus spp.	-0.65	0.05
Gram-negative bacteria	0.042	0.05

There were no significantly relationships between water parameters and the prevalence of *Legionella* because the positive samples of *Legionella* from total

examined samples were low (3.67%). Thus, susceptibility of *L. pneumophila* to chlorine was studied using the laboratory-adapted type strains.

The experiment used calcium hypochlorite as free chlorine residue to decontaminate *L. pneumophila*. The chlorine concentrations in this test were 0.1 to 0.5 mg/l and contact time were 0, 3, 6 and 24 h. The approximately 5×10^6 cells/ml of viable *L.pneumophila* in sterile tap water without other disinfectant were used as tested organisms. The effects of chlorine on *L. pneumophila* at various concentrations of chlorine and contact times were shown in Table 12.

Table 12 Viable *Legionella pneumophila* after exposed to chlorine

Chlorine concentration (mg/l)	Numbers of viable *L. pneumophila* at:			
	0 h	3 h	6 h	24 h
Control	3.5×10^6	2.0×10^6	2.3×10^6	3.5×10^6
0.1	2.0×10^6	0	0	0
0.2	2.3×10^6	0	0	0
0.3	3.5×10^6	0	0	0
0.4	2.0×10^6	0	0	0
0.5	2.0×10^6	0	0	0

Viable *L. pneumophila* were exposed to each of chlorine concentration (0.1-0.5 mg/l). No survivors were recovered after exposed at 3, 6 and 24 h. This result indicated the susceptibility of *L. pneumophila* to chlorine concentration at the standard criterion (0.1-0.5 mg/l) of tap water according to Metropolitan Waterworks Authority, Thailand.

4.7 Effects of pre-treatments on *Legionella pneumophila* biofilms

The experiments were done to determine the effect of 3 pre-treatment methods which were acid, heat and heat with 1% saponin addition on *L. pneumophila* in the biofilm samples. The recovery rates of *L. pneumophila* were shown in Table 13.

Table 13 Recovery rates of *L. pneumophila* in different methods

Pre-treatment methods	Mean viable *L. pneumophila* (CFU/ml)
Untreated	1.05×10^{6}
Acid pre-treatment	1.06×10^{5}
Heat pre-treatment	9.65×10^{4}
Heat + 1% saponin	1.12×10^{5}

The efficiency of acid pre-treatment, heat pre-treatment and heat with 1% saponin addition were not significantly difference ($p<0.05$).

4.8 Repeated cultivation after decontamination of *Legionella* spp. at the positive sites

The positive sample sites of *Legionella* spp. were reported to Suranaree Army Hospital for decontamination according to the recommendation of Department of Health, Ministry of Public Health. After 2 weeks, samples were collected and cultured again to prove that legionellae was destroyed completely or not.

After decontamination, *L. pneumophila* was not found but other microorganisms (heterotrophic bacteria and Gram-negative bacteria) still be found in those sites. *Pseudomonas* spp. was found in all of the decontaminated sites in both

water and biofilm samples. *Acinetobacter* could be detected again in only one biofilm sample. Heterotrophic bacteria densities were decreased in both water and biofilm samples. Coliform bacteria and *Staphylococcus* spp. were not found in both before and after decontamination. The results of this investigation were shown in Table 14.

Table 14 *L. pneumophila* and other microorganisms detected from tap water samples before and after decontamination in positive sample sites

Sample no.	Density of *L. pneumophila*		total plate count (CFU/ml)		Other microorganisms	
	before	**after**	**before**	**after**	**before**	**after**
Shower 4						
Water	0	0	6650	110	*Pseudomonas, Acinetobacter*	*Pseudomonas*
Biofilms	80 CFU/ml	0	21000	1560	*Pseudomonas, Acinetobacter, Alcaligenes*	*Pseudomonas, Acinetobacter*
Faucet 14						
Water	2 CFU/ml	0	3750	95	*Pseudomonas, Acinetobacter, Alcaligenes*	*Pseudomonas*
Biofilms	840 CFU/swab	0	62700	875	*Pseudomonas, Acinetobacter, Alcaligenes*	*Pseudomonas*
Faucet 15						
water	<1 CFU/ml	0	43000	100	*Pseudomonas, Acinetobacter, Flavobacterium*	*Pseudomonas*
biofilms	0	0	60000	1250	*Pseudomonas, Acinetobacter, Alcaligenes, Flavobacterium*	*Pseudomonas*

CHAPTER V

DISCUSSIONS

Epidemiological studies of *Legionella* spp. during outbreak investigations have established the role of aerosols produced by shower and tap faucets in disease transmission (Bollin *et al.*, 1985; Breiman *et al.*, 1990). Moreover, it has been suggested that dental lines may contribute legionellae to dental staffs and patients via respiratory tract (Atlas *et al.*, 1995; Walker *et al.,* 2000). Therefore, 109 samples from Suranaree Army Hospital were cultured for *Legionella* spp. and four of them (3.67%) were positive. The number of positive samples for *L. pneumophila* in this study was low when compared to the contamination of this organisms in the previous studies (Bollin *et al.,* 1985; Kusnetsov *et al.,* 2003; Leoni and Legnani, 2001; Lye *et al.,* 1997), which were 15-100% for legionellae contamination in potable water. However, the finding agreed with other studies in Thailand, which reported the prevalence of legionellae in potable water samples between 2.6-6% (นีรภา คงกันกง และคณะ, 2543; มณฑล เลิศคณาวานิชกุล, 2547) and <1-56% in environmental source and cooling tower samples (สมชัย บวรกิตติ, 2546; ทิพวรรณ กังแฮ และเกษร บุญยรักษ์โยธิน, 2547; วันทนา ปวีณกิตติพร และคณะ, 2547; Tishyadihigama *et al.*, 1995).

The positive results from Suranaree Army hospital suggest that Legionnaires' disease may possess some risk to patients or staffs in those wards. Since the samples were collected from medicine ward, GI tract microscopy room, emergency room and dental unit, therefore, the positive *L. pneumophila* might cause the nosocomial pneumonia in low immune patients (Breiman, 1993). The *L. pneumophila* positive sites were reported to the hospital for urgent decontamination to get rid of the microorganisms. Legionellae contaminated samples sites (faucets and showerheads) were decontaminated according to ASHRAE guideline 12-2000: Minimizing the risk of legionellosis associated with building water systems. The guideline recommended that the high-risk places, should monthly remove the showerheads and faucets to clean out sediment and scale then decontaminated them in chlorine bleach solution (ASHRAE, 2000). After decontamination, the water samples were repeated cultivation, legionellae was not found and other microorganisms were decreased. Ideally, hospital water systems should be *Legionella*-free. However, it is not known whether zero tolerance must be achieved, or some low level colonization may be acceptable without increasing the risk of nosocomial legionellosis (Ruef, 1998). However, the presence of legionellae in building water systems at hazard level 5 (≥100 CFU/ml according to Shelton *et al.* (1993) as mentioned in table 5.) is not necessary to be danger to the building occupants or associates with human disease because the water may not be aerosols and the distance from the disseminated source to exposed persons may not close enough to spread sufficient infectious dose for human infection (Bollin *et al.,* 1985; Shelton *et al.,* 1993).

In this experiment all of the positive samples were cultured by heat pre-treatment, acid pre-treatment and heat pre-treatment with 1% saponin addition (from

Quillaja saponaria, Sigma) on GVPC agar. The recovery of legionellae from environmental samples is often hampered by overgrowth of culture media by faster growing non-legionellae that may inhibit legionellae or may mask the presence of colonies on agar media (Lye *et al.*, 1997). Because legionellae have thermophilic characteristics and are relatively stable in acidic conditions, sample concentrates can be pretreated with heat or acid prior to culturing to inhibit background flora. If not carefully controlled however, these pre-treatment methods may also inhibit or injure legionellae (Bartie *et al*., 2003). Verran *et al.* (1995) showed that heat pre-treatment was more effective than acid pre-treatment at isolating *Legionella* spp. from environmental samples. But in this study, the positive numbers from environmental samples which were used to compare the pre-treatment effect were low. Thus the laboratory-adapted type strains of *L. pneumophila* were used to compare the effects of 3 types of pre-treatment (heat, acid and heat with 1% saponin) to the absence of pre-treatment. The numbers of organisms recovered after 3 pre-treatments were not significantly difference ($p<0.05$). Since the laboratory-adapted type strains of *L. pneumophila* were known to be more sensitive to pre-treatment conditions than environmental strains, these results might not necessarily be directly applicable to environmental samples (Bartie *et al.*, 2001). The effect of saponin on biofilm samples was also determined. Saponins are steroid or triterpenoid glycosides, common in a large number of plants and plant products that are important in human and animal nutrition. *Quillaja* saponins are non-ionic surfactants, resistant to salt, heat and extremely stable to acid pH. Main effects of *Quillaja* saponins are reduction of surface tension, formation of persistent foam and saponin-cholesterol complexes, emulsification of fats and oils, activation of cellular and humoral immune responses

and disruption of cell membranes (Francis *et al.,* 2002). The use of saponin in the previous study showed that it developed pores on the surface of virus. Saponin would lyse amoebic trophozoites in samples and release some viable intracellular *Legionella*. Amoeba named *Dictoyostelium discoideum* was lysed to release the intracellular bacteria by addition of 0.02% saponin (Solomon *et al.*, 2000). Thus, in this study, the biofilm samples were treated to determine the saponin's effect. The result showed that there was no observable difference in the microbial quantity between samples pre-heat treated with and without saponin. This study gave the same result as Benovic and Bentham (personal communication, February 21, 2002). It indicated that saponin did not cause biofilms to dissolve and release any *Legionella* which might be incorporated within them.

Many investigators indicated that culture method for the prevalence of legionellae from environmental sources gave the low efficiency of the recovery when compared with molecular techniques (Atlas, 1999). Numerous reasons were given such as laboratory media, stress of heat and acid pre-treatments, the antibiotic susceptibility, overgrowth by naturally occurring microorganisms, the presence of viable but nonculturable cells, and nutrient shock (Paszko-Kolva *et al.*, 1993). However, a recent study conducted by States *et al*. (1987) failed to recover *Legionella* species in municipal drinking water systems, despite the use of several isolation techniques. They believed that *Legionella* cells might be injured or inactivated by the presence of chlorine residual throughout the distribution system. Although, molecular techniques may solve the mentioned problems for legionellae recovery in the environmental samples, the cultural method remains to be the gold standard for the identification of legionellae. Moreover, CDC suggests that the routine environmental

culture of the hospital water supply for legionellae is an important strategy in prevention of nosocomial legionellosis and culture method is the method that can detect the viable organisms (Sabria and Yu, 2002).

In this study, the other microorganisms that found in the early samples were heterotrophic bacteria (mean 5.00×10^5 CFU/ml), *Staphylococcus* spp. (9.17%), *Pseudomonas* (89%), *Acinetobacter* (76.15%), *Alcaligenes* (57.80%), *Flavobacterium* (49.54%) but no coliforms. After decontamination, *Pseudomonas* and *Acinetobacter* were found. These bacilli could cause the nosocomial infections, especially, *Pseudomonas* spp. The patient might inhale the aerosols from sources (such as humidifiers of respirators) and the bacteria could bypass the normal respiratory defense mechanisms and initiate pulmonary infection (Ryan, 1984). Some viable bacteria had no health effects, variety of bacteria and bacterial concentration in water are the indicators of water system qualification. But heterotrophic bacteria contaminated in dental units were proposed as the serious conditions because the contaminated aerosols generated from dental equipments might exposed to people and increased the risk of infections to both patients and dental personnel (Atlas *et al.*, 1995; Walker *et al.*, 2000). From dental unit water systems (DUWS), all 9 samples from 3 dental unit sites carried more viable microorganisms than the maximum allowable load recommended by American Dental Association (ADA). Since the number of samples was not enough to summarize, therefore, the heterotrophic bacteria in dental lines should be further investigated as the serious vigilance. Because of the complex design of dental chair equipment, bacteria including *Legionella* spp. could survive within biofilms in the water lines stagnation (Atlas *et al*, 1995; Szymańska, 2004; Zanetti *et al.,* 2000).

The coliform group included species from the genera *Escherichia*, *Klebsiella*, *Enterobacter*, *Citrobacter* and *E. coli* were used as microbiological indicator for fecal contamination, which might potentially present the enteric pathogens in water. Although some coliforms are found in the intestinal tract of man, most are found throughout the environment and have little sanitary significance. The presence of coliform bacteria in tap water suggests that the treatment system is not working properly or there is a problem in the pipes. The enteropathogenic contaminations can cause diarrhea, nausea, vomiting and gastroenteritis. Usually, gastroenteritis is not serious for a healthy person, but it can lead to more serious problems for people with weakened immune systems, such as the young children, elder people, or immuno-compromised hosts (Geldreich, 1970).

In this study, the free chlorine concentrations of all collected samples were not detectable whereas the Metropolitan Waterworks Authority (MWA, 2004) recommended the standard level of chlorine in tap water at 0.2-0.5 mg/l. Among oxidizing agents, chlorine was known to be effective and widely used as disinfectant. Chlorine can be added to water using chlorine gas or hypochlorite salts (i.e., sodium or calcium hypochlorite). In water, chlorine exists as hypochlorous acid (HOCl), which has a pK_a of 7.6 at room temperature. This means that at $pH<7.6$ most of hypochlorous acid exists in its neutral form, HOCl, whereas at $pH>7.6$ most of hypochlorous acid exists as hypochlorite ion (OCl^-). Both HOCl and OCl^- are called free chlorine. HOCl is known to be more biocidal than OCl^-. Chlorine was reported that it effected the transport activities in bacteria leading to their inactivation (Kim *et al.*, 2002). Under practical conditions, bactericidal effectiveness of chlorine or others disinfectants cannot be based solely on results obtained from the laboratory.

Diverse physical, chemical, and biological conditions that may exist in the distribution of the water systems, these conditions can markedly affect to the bactericidal action (Skaliy *et al.*, 1980). This study showed that *L. pneumophila* was highly susceptible to free chlorine using in municipal tap water. However, chlorine is unstable in the presence of organic matters and may rapidly neutralize (Skaliy *et al.*, 1980). To maintain the recommended levels of free chlorine may be required for decontamination. On the other hand, high chlorine concentration is corrosive to the pipe system (Kim *et al.*, 2002). In this study, the long time of storage water in a storage tank before distributed to the other sites of hospital might be the reason of low chlorine concentration which was one cause for the bacterial contamination in tap water. From this study, chlorine at 0.1 mg/l which was similar to concentration that might be found in the distribution systems showed bactericidal action against 5×10^6 cells/ml of *L. pneumophila* in water at $\geq$3 h exposure. Kuchta *et al.* (1983) found that at the same chlorine concentration, 99% of 3×10^4 cells/ml of *L. pneumophila* was killed within 40 min. For biofilm decontamination, many investigators (Green, 1993; Muraca *et al.*, 1987; Skaliy *et al.*, 1980) suggested that >3 mg/l of free chlorine was needed to penetrate the biofilms and kill the bacteria included *Legionella* spp.

The low concentration of free chlorine contained in water samples from Suranaree Army hospital might be the risk for the pathogenic contaminations. Thus, staffs should obtain the level of free chlorine residue at 0.2-0.5 mg/l for decontaminated the microorganisms at the safety level recommended by MWA. Routine environmental culture of the hospital water supply for legionellae should be examined to prevent nosocomial legionellosis. Especially the dental lines which might contribute legionellae to patients and dental staffs via respiratory tract. Since higher

rates of seropositivity for *Legionella* antibodies have been found among dental personnels than among the general publics, suggesting that aerosols generated in dental operatories are the source of exposure to legionellae (Atlas *et al.*, 1995; Kettering *et al.*, 2002; Souza-Gugelmin *et al.*, 2003). The results of this study gave immediately benefit to the patients and personnel in the public health community not to confront to the risk of nosocomial infections.

CHAPTER VI

CONCLUSIONS

Legionella especially *Legionella pneumophila* are recognized as opportunistic pathogens of human causing nosocomial pneumonia. They have a worldwide distribution and associate with aquatic environments. Routine environmental culture of the hospital water supply for legionellae has proven to be an important strategy in prevention of nosocomial legionellosis. The primary mode of legionellosis is inhalation of *Legionella* organisms in aerosolized droplets. Transmission occurs occasionally via other routes, including direct inoculation of surgical wounds with contaminated potable water during placement of surgical dressing, aerosolization of contaminated warm potable water via showerheads or tap water faucets and also respiratory care equipments. Moreover, seropositivity for *Legionella* antibodies have been found among dental personnel than among the general public, suggesting that aerosols generated in dental operatories are the source of exposure to *Legionella* spp. Therefore, 109 municipal tap water samples from showerheads and faucets from medicine ward, GI tract microscopy room, emergency room and dental unit of Suranaree Army Hospital were cultured for *Legionella* spp. Four of them (3.67%) were positive for *Legionella* and were identified as *L. pneumophila* by positive result for hippurate hydrolysis reaction. The number of positive samples for *L. pneumophila* in this study was likely the same as other studies in Thailand (2.6-6%). The *L. pneumophila* positive sites were reported to the hospital for urgent decontamination

to get rid of the microorganisms. Legionellae contaminated samples sites were decontaminated and the water samples were repeated cultivation. Legionellae was not found and other microorganisms were decreased. The other microorganisms that found in the early samples were heterotrophic bacteria (mean 5.00×10^5 CFU/ml), *Staphylococcus* spp. (9.17%), *Pseudomonas* (89.00%), *Acinetobacter* (76.15%), *Alcaligenes* (57.80%), *Flavobacterium* (49.54%) but no coliforms. After decontamination, *Pseudomonas* and *Acinetobacter* were found. These bacteria could cause the nosocomial infections, especially, *Pseudomonas* spp. The patients might inhale the aerosols from source (such as humidifiers of respirators) and the bacteria could bypass the normal respiratory defense mechanisms and initiate pulmonary infection. From dental unit water systems (DUWS), all 9 samples from 3 dental unit sites carried more viable microorganisms than the maximum allowable load recommended by American Dental Association (ADA). Since the number of samples was not enough to summarize, therefore, the heterotrophic bacteria in dental lines should be further investigated as serious vigilance. In this study, the positive numbers from environmental samples which were used to compare the pre-treatment effect were low. Thus the laboratory-adapted type strains of *Legionella* were used to compared the effects of 3 types of pre-treatment (heat, acid and heat with 1% saponin). The numbers of organisms recovered after 3 pre-treatments were not significantly difference ($p<0.05$). The effect of saponin on biofilm samples was also determined. The result showed that there was no observable difference in the microbial quantity between samples pre-heat treated with and without saponin. It indicated that saponin did not cause biofilms to dissolve and release any *Legionella* which might be incorporated within them. The free chlorine concentrations of all

collected samples were not detectable whereas the Metropolitan Waterworks Authority (MWA) recommended the standard level of tap water at 0.2-0.5 mg/l. The low concentration of free chlorine contained in water samples from Suranaree Army Hospital might be the risk for the pathogenic contaminations. Thus, staffs should obtain the level of free chlorine residue at the safety level recommended by MWA. Routine environmental culture of the hospital water supply for legionellae should be examined to prevent nosocomial legionellosis. The results of this study gave immediately benefit to patients and personnel in the public health community not to confront to the risk of nosocomial infections.

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APPENDICES

APPENDIX A

MICROBIOLOGICAL MEDIA

1. Buffered charcoal yeast extract alpha base (BCYE)

Charcoal	2.0	g
Yeast extract	10.0	g
ACES buffer	10.0	g
Alpha-ketoglutarate	1.0	g
Ferric pyrophosphate soluble	0.25	g
L-cysteine, HCl.H_2O	0.4	g
Agar	15.0	g

Final pH 6.9 (± 0.2)

Preparation of medium: dissolved charcoal, yeast extract, ACES buffer, alpha-ketoglutarate and agar in 1 l distilled water, adjusted pH to 6.9 with 0.1 N KOH and heated to boil. Then, sterilized by autoclaving at 121°C for 15 min. Dissolved 0.4 g L-cysteine and 0.25 g ferric pyrophosphate in 10 ml of water each and filter sterile separately. After agar base was cooled, added L-cysteine and ferric pyrophosphate in that order and dispensed into sterilize petri dishes.

2. Glycine vancomycin polymyxin B cyclohexamide medium (GVPC)

Glycine	3.0	g
Polymyxin B	100	units/ml
Vancomycin	5	µg/ml
Cyclohexamide	80	µg/ml

Preparation of medium: to cooled BCYE-alpha base with glycine, add filter-sterilized antibiotics and mix. The medium was dispensed into sterilized petri dishes.

3. Baird-Parker agar base

Tryptone	10.0	g
Beef extract	5.0	g
Yeast extract	1.0	g
Glycine	12.0	g
Sodium pyruvate	10.0	g
Lithium chloride	5.0	g
Agar	15.0	g

Final pH 7.0 (± 0.2)

Preparation of medium: all components were added to distilled water and brought volume up to 1 l. The medium was mixed thoroughly and gently heated until dissolved. The medium was autoclaved at 121°C for 15 min. After agar base was cooled to 50°C, added 50ml egg yolk tellurite/l, mixed well and dispensed into sterilize petri dishes.

4. MacConkey agar

Peptone	17.0	g
Protease peptone	3.0	g
Lactose	10.0	g
Bile salts	1.5	g
Sodium chloride (NaCl)	5.0	g
Neutral red	0.03	g
Crystal violet	0.001	g
Agar	15.0	g

Final pH 7.1 (± 0.2)

Preparation of medium: all components were added to distilled water and brought volume up to 1 l. The medium was mixed thoroughly and gently heated until dissolved. The medium was autoclaved at 121°C for 15 min. Dispensed into sterilize petri dishes.

5. M-Endo medium

Tryptose or poly peptone	10.0	g
Thiopeptone or thiotone	5.0	g
Casitone or tryticase	5.0	g
Yeast extract	1.5	g
Lactose	12.5	g
Sodium chloride (NaCl)	5.0	g
Dipotassium hydrogen phosphate (K_2HPO_4)	4.375	g
Potassium dihydrogen phosphate (KH_2PO_4)	1.375	g

Sodium lauryl sulfate	0.05	g
Sodium desoxycholate	0.10	g
Sodium sulfite (Na_2SO_3)	2.10	g
Basic fuchsin	1.05	g

Final pH 7.1 (± 0.2)

Preparation of medium: rehydrated in 1l. water containing 20 ml of 95% ethanol. Heated to near boiling, then promptly removed from heat and cooled to between 45-50°C. Kept into sterile container in the dark and stored at 4-8°C. Do not sterilized by autoclaving.

6. Plate count agar (Tryptone glucose yeast agar)

Tryptone	5.0	g
Yeast extract	2.5	g
Glucose	1.0	g
Agar	15.0	g

Final pH 7.0 (± 0.2)

Preparation of medium: all components were added to distilled water and brought volume up to 1 l. The medium was mixed thoroughly and gently heated until dissolved. The medium was autoclaved at 121°C for 15 min. Dispensed into sterilize petri dishes.

APPENDIX B

CHEMICAL REAGENTS

1. Acid treatment reagent (0.2 M KCl/HCl)

Solution A: 0.2 M KCl (14.9 g/l in distilled water).

Solution B: 0.2 M HCl (16.7 ml/l 10N HCl in distilled water).

Preparation of reagent: mixed 18 parts of solution A with 1 part of solution B. Check pH against a pH 2.0 standard buffer and sterilize by autoclaving at 121°C for 15 min.

2. Alkaline neutralizer reagent (0.1 M KOH)

Potassium hydroxide (KOH) 6.46 g

Preparation of reagent: the component was added to deionized water, mixed thoroughly until dissolved and brought volume up to 1 l as stock solution. Diluted 10.7 ml of stock solution with 100 ml deionized water and sterilized by autoclaving at 121°C for 15 min.

3. 0.1 N Sodium thiosulfate ($Na_2S_2O_3$)

Sodium thiosulfate ($Na_2S_2O_3 \cdot 5H_2O$) 24.82 g

Preparation of reagent: the component was added to distilled water, mixed thoroughly until dissolved and brought volume up to 1 l. The reagent was autoclaved at 121°C for 15 min.

4. 1% Hippurate reagent

Sodium hippurate	0.1	g

Preparation of reagent: the component was added to sterile distilled water, mixed thoroughly until dissolved and brought volume up to 10 ml. The reagent was dispensed for 0.4 ml in microcentrifuge and stored at –20°C.

5. 3.5% Ninhydrin

Ninhydrin	0.35	g
1-Butanol	5.0	ml
Acetone	5.0	ml

Preparation of reagent: 1-butanol and acetone were mixed then added ninhydrin and mixed thoroughly until dissolved. The reagent was stored in brown bottle.

APPENDIX C

STANDARD OF TAP WATER RECOMMENDED BY METROPOLITAN WATERWORKS AUTHORITY (BASED ON WHO GUIDANCE 1993)

Table 1 C Standard of tap water recommended by Metropolitan Waterworks Authority (based on WHO guidance 1993)

Parameters	Units	Recommend
1. Bacteriology quality		
Total coliform bacteria	MPN/100 ml	None
E. coli	MPN/100 ml	None
2. Physical and chemical quality		
Appearance color	True color unit	15
Turbidity	NTU	5
Taste and odor	-	
Arsenic	mg/l	0.01
Cadmium	mg/l	0.003
Chromium	mg/l	0.05
Cyanide	mg/l	0.07
Lead	mg/l	0.01
Fluoride	mg/l	1.5
Chloride	mg/l	250

Table 1 C (continue).

Parameters	Units	Recommend
Copper	mg/l	1
Iron	mg/l	0.3
Manganese	mg/l	0.1
Aluminium	mg/l	0.2
Sodium	mg/l	200
Sulfate	mg/l	250
Zinc	mg/l	3
Hydrogen sulfide	mg/l	0.05
Total dissolved solids	mg/l	1000
Nitrate as N	mg/l	10
Ammonia as N	mg/l	1.5
Benzene	mg/l	10
Carbon tetrachloride	mg/l	2
Dichloromethane	mg/l	20
1, 2-Dichloroethane	mg/l	30
Benzo(a)pyrene	mg/l	0.7
3. Pesticides		
Aldrin/Dieldrin	mg/l	0.03
Chlordance	mg/l	0.2
DDT	mg/l	2
2, 4-D	mg/l	30

Table 1C (continue).

Parameters	Units	Recommend
Heptachlor and Heptachlor epoxide	mg/l	0.03
Hexachlorobenzene	mg/l	1
Lindane	mg/l	2
Methoxychlor	mg/l	20
Pentachlorophenol	mg/l	9
4. Trihalomethanes		
Chloroform ($CHCl_3$)	mg/l	200
Bromodichloromethane ($CHBrCl_2$)	mg/l	60
Dibromochloromethane ($CHBr_2Cl$)	mg/l	100
Bromoform ($CHBr_3$)	mg/l	100
5. Radioactive		
Gross alpha activity	Bq/l	0.1
Gross beta activity	Bq/l	1

Source: Metropolitan Waterworks Authority (2004).

APPENDIX D

HAZARD LEVEL AND REMEDIAL ACTIONS CRITERIA FOR LEGIONELLAE

Table 1 D Quantitative criteria for viable legionellae

Legionellae/ml	Hazard level		
	Cooling towers and evaporative condensers	Potable water	Humidifier/fogger
Detectable, but<1	1	2	3
1-9	2	3	4
10-99	3	4	5
100-999	4	5	5
≥1000	5	5	5

Source: Shelton *et al.*, 1993.

Table 2 D Remedial actions

Hazard level	Actions
1	Review routine maintenance program recommended by the manufacturer of the equipment to ensure that the recommended program is being followed. The presence of barely numbers of legionellae represents a low level of concern.
2	Implement action 1. Conduct follow-up analysis after a few weeks for evidence of further *Legionella* amplification. This level of legionellae represents little concern, but the number of organisms detected indicates that the system is a potential amplifier for legionellae.
3	Implement action 2. Conduct review of premises for direct and indirect bioaerosol contact with occupants and health risk status of people who may come in contact with the aerosol. Depending on the results of the review of the promises, action related to cleaning and /or biocide treatment of the equipment may be indicated. This level of legionellae represents a low but increased level of concern.
4	Implement action 3. Cleaning and/or biocide treatment of the equipment is indicated. This level of legionellae represents a moderately high level of concern, since this approaching level that may cause outbreaks. It is uncommon for samples to contain numbers of legionellae that fall in this category.

Table 2 D (continue).

Hazard level	Actions
5	Immediate cleaning and/or biocide treatment of the equipment is definitely indicated. Conduct post treatment analysis to ensure effectiveness of the corrective action. The level of legionellae represents a high level of concern, since it poses the potential for causing an outbreak. It is very uncommon for samples to contain numbers of legionellae that fall in this category.

Source: Shelton *et al*., 1993.

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