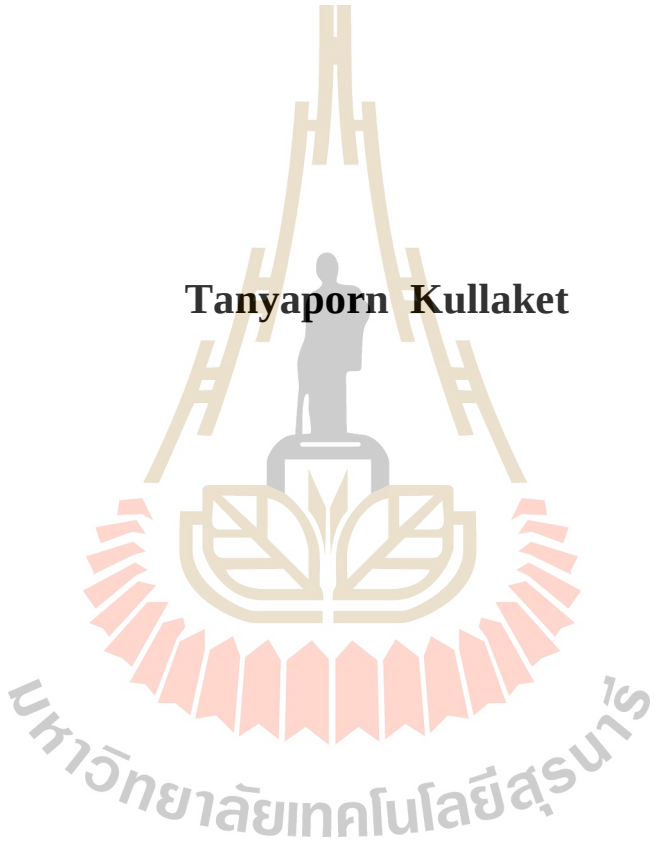
DETECTION OF LEGIONELLA spp. AND OTHER PATHOGENS IN WATER SYSTEMS OF NURSING HOMES AND SPA POOLS

Tanyaporn Kullaket



A Thesis Submitted in Partial Fulfillment of the Requirements for the Degree of Master of Science in Microbiology

Suranaree University of Technology

Academic Year 2017

การตรวจหาเชื้อ *Legionella* spp. และจุลินทรีย์ก่อโรค ในระบบน้ำใช้ของสถานพยาบาลพักฟื้นและสระน้ำสปา

นางสาวธันยาภรณ์ กุลเกษ

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
สาขาวิชาจุลชีววิทยา
มหาวิทยาลัยเทคโนโลยีสุรนารี
ปีการศึกษา 2560

DETECTION OF *LEGIONELLA* spp. AND OTHER PATHOGENS IN WATER SYSTEMS OF NURSING HOMES AND SPA POOLS

Suranaree University of Technology has approved this thesis submitted in partial fulfillment of the requirements for a Master's Degree.

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ธันยาภรณ์ กุลเกษ : การตรวจหาเชื้อ *Legionella* spp. และจุลินทรีย์ก่อโรคในระบบน้ำใช้ของสถานพยาบาลพักฟื้นและสระน้ำสปา (DETECTION OF *LEGIONELLA* spp. AND OTHER PATHOGENS IN WATER SYSTEMS OF NURSING HOMES AND SPA POOLS) อาจารย์ที่ปรึกษา : รองศาสตราจารย์ ดร.ทัศนีย์ เสาวนะ, 90 หน้า

เชื้อ *Legionella* spp. เป็นแบคทีเรียแกรมลบ ไม่สร้างสปอร์ สปีชีส์ก่อโรคของจีนัสนี้คือ *Legionella pneumophila* ทำให้เกิดโรค Legionellosis และ Pontiac fever กลุ่มคนที่เสี่ยงต่อการติดเชื้อคือ ผู้สูงอายุ ผู้ที่สูบบุหรี่ และผู้ป่วยที่มีภูมิคุ้มกันต่ำ ดังนั้นจุดประสงค์ของการศึกษาในครั้งนี้คือ การตรวจหาเชื้อ *Legionella* spp. และจุลินทรีย์ก่อโรคในระบบน้ำใช้ของสถานพักฟื้นคนชราและสระน้ำสปา การเก็บตัวอย่างน้ำได้ทำการเก็บจากสถานพักฟื้นคนชรา 30 ตัวอย่างและจากสระน้ำสปาอีก 30 ตัวอย่าง ในบริเวณกรุงเทพมหานครและจังหวัดนครราชสีมา ทำการตรวจหาเชื้อ *Legionella pneumophila* และเชื้อจุลินทรีย์ก่อโรคอื่น ๆ โดยวิธีการเพาะเลี้ยงเชื้อและวิธีทางชีวเคมี เชื้อ *Legionella* ถูกพบในระบบของน้ำของสถานพักฟื้นคนชรา ร้อยละ 16.67 และร้อยละ 43.33 ในสระน้ำสปา ส่วนเชื้อแบคทีเรียอื่น ๆ ที่พบในสถานพักฟื้นคนชรา คือ *Pseudomonas* spp. (ร้อยละ 33.33) *Enterobacter* spp. (ร้อยละ 13.33) *Acinetobacter* spp. (ร้อยละ 16.67) *Escherichia coli* (ร้อยละ 10) Coliform (ร้อยละ 20) และ *Staphylococcus* spp. (ร้อยละ 30) และเชื้อแบคทีเรียที่พบในสระน้ำสปา คือ *Pseudomonas* spp. (ร้อยละ 36.67) *Enterobacter* spp. (ร้อยละ 3.33) *Citrobacter* spp. (ร้อยละ 10) Coliform (ร้อยละ 13.33) และ *Staphylococcus* spp. (ร้อยละ 3.33) ส่วนเชื้อ *Escherichia coli* พบเฉพาะในสถานพักฟื้นคนชราเท่านั้น หลังจากการให้ความรู้และแนะนำให้ทำการกำจัดเชื้อ *Legionella* แล้ว ทำการเก็บตัวอย่างมาเพาะเชื้ออีกครั้ง ในสถานพักฟื้นคนชรา ไม่พบเชื้อ *Legionella* ส่วนในสระน้ำสปาเชื้อ *Legionella* มีจำนวนลดลงเหลือ ร้อยละ 23.33 และเชื้อจุลินทรีย์อื่น ๆ ไม่พบยกเว้น *Pseudomonas* spp. *Enterobacter* spp. และ *Escherichia coli*

จากผลการศึกษาแสดงให้เห็นถึงการแพร่กระจายของเชื้อ *Legionella* spp. และเชื้อก่อโรคอื่น ๆ จึงควรตระหนักถึงความเป็นไปได้ในการระบาดของเชื้อ *Legionella* spp. และเชื้อแบคทีเรียก่อโรคอื่น ๆ ในระบบน้ำใช้ของสถานพักฟื้นคนชราและสระน้ำสปาที่จะมีผลต่อผู้มีภูมิคุ้มกันต่ำด้วย

สาขาวิชาจุลชีววิทยา ลายมือชื่อนักศึกษา ธันยาภรณ์ กุลเกษ

ปีการศึกษา 2560 ลายมือชื่ออาจารย์ที่ปรึกษา

TANYAPORN KULLAKET : DETECTION OF *LEGIONELLA* spp. AND OTHER PATHOGENS IN WATER SYSTEMS OF NURSING HOMES AND SPA POOLS. THESIS ADVISOR : ASSOC. PROF. TASSANEE SAOVANA, Ph.D. 90 PP.

LEGIONELLA spp./ OTHER PATHOGENS/ WATER SYSTEMS/ NURSING HOMES/ SPA POOLS

Legionella spp. is gram-negative and non-spore-forming bacteria. The infective species of this genus is *Legionella pneumophila* that can cause the Legionellosis and Pontiac fever. The people at risk of infection are the elderly, smokers and the immunosuppressed patients. So, the objectives of this study were to detect *Legionella* spp. and other bacterial pathogens in water systems of nursing homes and spa pools. The water samples were collected from 30 nursing homes and 30 spa pools in Bangkok and Nakhon Ratchasima provinces and examined for the presence of *Legionella pneumophila* and other bacterial pathogens by culture methods and biochemical methods. The *Legionella* spp. was found 16.67% and 43.33% in the water systems of nursing homes and of spa pools, respectively. Other bacteria in nursing homes were *Pseudomonas* spp. (33.33%), *Enterobacter* spp. (13.33%), *Acinetobacter* spp. (16.67%), *Escherichia coli* (10%), Coliform (20%), and *Staphylococcus* spp. (30%). The other bacteria in spa pools were *Pseudomonas* spp. (36.67%), *Enterobacter* spp. (3.33%), *Citrobacter* spp. (10%), Coliform (13.33%), and *Staphylococcus* spp. (3.33%). The *Escherichia coli* was found in nursing homes only. After education and advice about the decontamination of *Legionella*, samples from decontaminated sites

were repeated cultivation. The *Legionella* was not found in nursing homes. The *Legionella* in the spa pools was decreased to 23.33% and other microorganisms were not found, except for *Pseudomonas* spp., *Enterobacter* spp. and *Escherichia coli*. The results of this study showed the epidemiology of *Legionella* spp. and other pathogens which must be concerned about the possible outbreak of these species in water systems of nursing homes and spa pools that will affect to the low immunity people.

School of Microbiology Student's Signature Tanyaporn Kullaket

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LIST OF ABBREVIATIONS

%	Percent
BCYE	Buffered Charcoal Yeast Extract Agar
°C	Degree Celsius
CFU/ml	Colony Forming Units per milliliter
DFA	Direct fluorescence antibody
DNA	Deoxyribonucleic acid
ELISA	Enzyme-linked immunosorbent assay
et al.	et alia (and other)
(m, μ) g	(milli, micro) Gramme
(μ) g/l	(micro) Gramme per litre
GVPC	Glycine Vancomycin Polymyxin B and Cycloheximide Agar
IFA	Indirect fluorescent antibody
(m, μ)l	(milli, micro) Litre
M	Molar
ml/l	Millilitre per litre
min	Minute
MWA	Metropolitan Waterworks Authority, Thailand
PCR	Polymerase chain reaction
sp., spp.	Species (singular, plural)

LIST OF ABBREVIATIONS (Continued)

rRNA	Ribosomal ribonucleic acid
WHO	World Health Organization

CHAPTER I

INTRODUCTION

1.1 Background/Problems

The water distribution system in many buildings such as hospital, nursery, nursing home or even swimming pool and spa are very important because it may be a source of infections, especially respiratory infections that are caused by the inhalation of bacteria contaminated by water aerosols. The contaminated aerosols that are generated by respiratory equipments, including humidifiers and nebulizers have been reported that they can transmit airborne pathogens into the respiratory tract of patients (Woo, Goetz and Yu, 1992). One of bacterial pathogens that cause respiratory disease is Legionellae. They are gram-negative and non-spore-forming bacteria. These bacteria are short rod-shaped cells and are described as coccobacillary (Rodgers, Macrae and Lewis, 1978). The representative species of the genus is Legionella pneumophila that can cause the Legionellosis (Percival and Williams, 2014). Legionellae are commonly found in natural water environments (e.g., rivers, lakes, lagoon and reservoirs) and human-made water systems (e.g., cooling tower, water heater tanks, fountains and spa pools). The water distribution system that is not appropriately managed can act as the source of major outbreaks of Legionellosis (Moore and Walker, 2014). People at risk are the elderly, smokers and the immunosuppressed patients.

The Legionellosis is divided into two distinct clinical entities, Pontiac fever is a self-limited flu-like illness and has a high rate of infection of about 95% and Legionnaires' disease which is a severe multisystem disease involving pneumonia with about 5%, rate of infection but symptoms are more severe than Pontiac fever and may lead to death (Fields, Benson and Besser, 2002). In the United Kingdom, Legionnaires' disease caused by L. pneumophila, is rare but serious disease. Between 2009 to 2011, there were 934 confirmed cases in England and Wales, 355 (38%) affected persons occurred diseases while they were travelling abroad (Moore and Walker, 2014). In the water system of nursing home such as one in Iran, Legionella were found 18.2% from 77 samples (Ahmadinejad, Shakibaie, Shams and Khalili, 2011). In year 1990, nursing home in Slovenia found 15 Legionella infected cases from 234 patients (Skaza, Beskovnik, Storman, Kese and Ursic, 2012). The water systems of hot spring, spa, swimming pool or public baths in Taiwan, 20 Legionella cases were found from 72 samples, representing 27.8% from all samples (Huang et al., 2010). In Thailand, between 1984 to 2002, there were 17 patients reported to be infected with Legionella. Fourteen patients were infected by L. pneumophila, two patients were infected by Legionella spp. and another one was infected by L. jordanis. Legionella spp. have been isolated from human-made water systems and environmental samples in several regions of Thailand (Bovornkitti, 2010). During 2006 to 2007 Legionella occurred in travellers in Phuket province. Total 5 confirmed cases and 1 presumptive case were detected among all Scandinavians staying at the hotel in Phuket province. The risk factors of infection were showers in the hotels which had Legionella and people aged more than 45 years old had increased risk for Legionella spp. infection (Buathong et al., 2013).

Other microorganisms that may be found in the water systems and cause problems to human are gram-negative bacteria that are commonly found in soil, water and natural environments and may be found in the hospitals causing nosocomial infections. Most frequently reported microorganisms are Enterobacteriaceae, Pseudomonas aeruginosa, Staphylococcus aureus, coagulase-negative staphylococci and fungi which include Flavobacterium, Alcaligenes and Acinetobacter (Vincent et al., 1995). They cause many diseases and may be the causes of death.

From the above data, it is necessary to study the incident of Legionella spp. and other microorganisms in water systems of nursing homes and spa pools for more information. These results will stimulate the staffs to aware since it may affect anyone who concerns with the water distribution systems.

The purpose of this work was to study the prevalence of legionellae and other bacterial pathogens in water systems of nursing homes and spa pools and the result would make nursing homes and spa staffs to concern the possible outbreak of Legionellosis and other bacterial pathogens.

CHAPTER II

LITERATURE REVIEW

2.1 Legionella species

Legionella is the gram-negative bacteria which is short rod shape (approximately 0.3–0.9 mm wide and 1–3 mm long) and non-spore-forming. Legionella is the single genus of the family Legionellaceae. It comprises at least 50 species and is subdivided into 70 distinct serogroups (Table 1). Legionella pneumophila (serogroup 1) is the most common and be the major genus that causes the disease. Legionella is the aerobic and fastidious bacteria which its nature will not grow on traditional bacteriological media but it requires an enriched medium supplemented with L-cysteine and ferric salts. The optimal growth temperature for Legionella is 35 °C. Legionella is catalase-positive and unable to reduce nitrate. This bacterium also does not utilize carbohydrates by either oxidation or fermentation (Percival and Williams, 2014).

Table 1 Legionella species with standing in nomenclature.

Species	First Isolated From	Human pathogen
L. adelaidensis	Cooling tower water	
L. anisa	Potable water and a cooling tower	Yes
L. beliardensis	Water in France	
L. birminghamensis	Cardiac transplant recipient	Yes
L. bozemanae	Respiratory specimen	Yes
L. brunensis	Cooling tower water	
L. busanensis	Cooling tower water in Korea	
L. cardiaca	Isolated from a case of native valve endocarditis	Yes
L. cherrii	Water, thermally altered	
L. cincinnatiensis	Pneumonia patient	Yes
L. drancourtii	Water in UK	
L. dresdenensis	River wate	
L. drozanskii	Isolated via amoebal enrichment from various sources in the UK	
L. dumoffii	Respiratory specimen	Yes

Table 1 (Continued) Legionella species with standing in nomenclature.

Species	First Isolated From	Human pathogen
L. erythra	Water, cooling tower	
L. fairfieldensis	Cooling tower water in Australia	
L. fallonii	Isolated via amoebal enrichment from various sources in the UK	
L. pneumophila	Pneumonia patient	Yes
L. quateirensis	Water, shower in bathroom	
L. quinlivanii	Water	Yes
L. rowbothamii	Isolated via amoebal enrichment from various sources in the UK	
L. rubrilucens	Tap water	Yes
L. sainthelensi	Water near Mt. St. Helens	Yes
L. santicrucis	Tap water	
L. shakespearei	Water, cooling tower	
L. spiritensis	Water, lake	
L. steelei	Human respiratory specimen	

Table 1 (Continued) Legionella species with standing in nomenclature.

Species	First Isolated From	Human pathogen
L. steigerwaltii	Tap water	
L. taurinensis	Water in Italy	
L. tucsonensis	Human, renal transplant recipient	
L. tunisiensis	Environmental water	
L. wadsworthii	Pneumonia patient	Yes
L. waltersii	Water in Australia	Yes
L. worsleiensis	Return flow of cooling tower water	Yes
L. yabuuchiae	Soil contaminated with industrial wastes in Japan	

Source : (Nazarian, De Jesus and Musser, 2015).

2.2 Legionella ecology

Legionella pneumophila is found in the natural aquatic environment and this bacterium is capable to survive in the extreme ranges of the environmental conditions (Fliermans et al., 1981). The natural reservoirs of Legionella are freshwater systems such as rivers, lakes or thermal waters. Apart from their natural habitat, Legionella bacteria is also able to colonize in the man-made water systems such as air cooling towers, conditioning systems, hot water systems, vegetable misters, whirlpools and dental-unit water lines (Guyard and Low, 2011). Although, Legionella can be found in water ranging from cold to very hot, its multiplication is restrictive to temperature between 25-42 °C with an optimal growth at 35 °C (Fields, 2008) and does not

multiply at temperature below 20 °C. The Legionella can survive as intracellular parasites of protozoa, amoebae, ciliated or slime moulds, when the temperature of aquatic environments changes, it can shift the balance between protozoa and bacteria, resulting in rapid multiplication of Legionellae, which is the etiology of the human disease.

Some outbreaks of Legionellosis associated with construction, and can be transmitted to humans via soil or containing microorganism by not washing hands after gardening. However, L. pneumophila does not survive in dry environments and the outbreaks are more likely the result of massive descalement of plumbing systems due to changes in water pressure during construction (Fields, Benson and Besser, 2002).

2.3 Pathogenesis

Legionella pneumophila serogroup 1 is the most virulent Legionella species and the most common cause of disease. The infection of Legionella is commonly found through inhalation of contaminated aerosols produced by water systems such as cooling towers, showers and faucets. Other modes of transmission of Legionella are respiratory tract manipulations. Person to person transmission has not been reported both of Pontiac fever and Legionnaires' disease (Guyard and Low, 2011). The Legionella can be found naturally in freshwater and acts as a parasite of amoebae. If inhaled into the lung, Legionella can replicate within the alveolar macrophages (Swanson and Hammer, 2000).

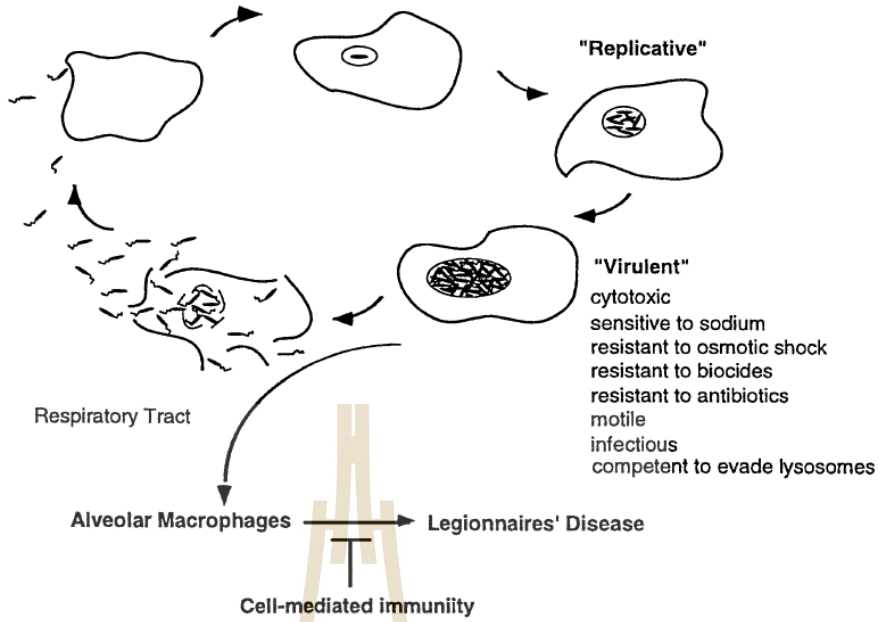

Figure 1 Legionella pneumophila life cycle in amoebae (Swanson and Hammer, 2000).

The pathology of Legionellosis is similar to all Legionella spp. There are heavy inflammatory infiltrations including neutrophils and macrophages, abscess formation, necrosis, inflammation of small blood vessels (Lau and Ashbolt, 2009) and other clinical symptoms such as pneumonia.

2.4 Legionellosis

Legionellosis is the disease caused by Legionella. Over 90% of cases of Legionellosis are caused by L. pneumophila and other species include L. longbeachae, L. feeleii, L. micdadei and L. anisa which are the causative agents of a less severe infection known as Pontiac fever. The Legionellosis is divided into two distinct clinical entities, Legionnaires' disease, a severe multisystem disease involving pneumonia and Pontiac fever, a self-limited flu-like illness (Fields, Benson and Besser, 2002). The incubation period for Legionnaires' disease is typically 2–14 days,

with the infection lasting weeks to months. In Pontiac fever, symptoms include fever, chills, myalgia and headache. The incubation period for Pontiac fever is 5–66 hours and symptoms last for 2–7 days (Percival and Williams, 2014). The Legionnaires'disease presents with a broad spectrum of illness, ranging from a mild cough and low-grade fever to stupor, respiratory failure, and multiorgan failure. In the early illness, patients have nonspecific symptoms including fever, malaise, myalgias, anorexia, and headache (Table 2). The temperature often exceeds 40 °C (Stout and Yu, 1997).

Table 2 Symptoms associated with Legionellosis.

Legionnaires' disease
Mild cough to a raidly fatal pneumonia. Death occurs through progressive pneumonia with respiratory failure and/or shock, acute kidney and multi-organ failure
Incubation period: 2-10 days (up to 16 days in recent outbreaks)
- Fever
- Headache
- Loss of appetite
- Malaise
- Lethargy
In some cases:
- Diarrhea
- Muscle pain
- Confusion

Table 2 (Continued) Symptoms associated with Legionellosis.

- Initial mild cough
- Phlegm (up to 50% of patients)
- Blood-streaked phlegm or hemoptysis (1/3 of the patients)
Pontiac fever
Acute self-limiting influenza-like illness lasting 2-5 days
Incubation period: few to 48 h.
- Fever
- Chills
- Headache
- Malaise
- Myalgia
- Not fatal

Source: (Percival and Williams, 2014)

The Legionnaires'disease can generate multilobar in the lungs, with focal or lobar consolidation presenting as either red or grey hepatization. Acute renal failure, shock, disseminated intravascular coagulation, coma, respiratory insufficiency and circulatory collapse are the major factors associated with death (Percival and Williams, 2014).

There are many risk factors that cause Legionellosis such as the people aged 50 years old or over, smoking or having smoked heavily in the past, drinking alcohol heavily, including people who have an underlying medical conditions, such as diabetes, kidney disease or a pre-existing lung condition and having a weak immune system for example, people with AIDS or cancer.

For the treatment of Pontiac fever, treatment does not use antibiotics because it is a self-limited illness and recovery usually occurs within 1 week. The Legionnaires disease is treated with antibiotics, the two most potent classes of antibiotics are the macrolides (azithromycin) and the quinolones (ciprofloxacin, levofloxacin, moxifloxacin, gemifloxacin, trovofloxacin). Other agents that have been shown to be effective include tetracycline, doxycycline, minocycline, trimethoprim and sulfamethoxazole. Macrolides (azithromycin) is the drug of choice for children with suspected or confirmed Legionnaires'disease and quinolones (levofloxacin, moxifloxacin) are recommended for adults with severe disease. Both of antibiotics are highly effective and have few side-effects more than other drugs, so, they become to be antilegionella drugs in healthy and immunocompromised individuals. The recommended duration of therapy is 5-14 days if azithromycin is used. For the patients with severe disease or immunocompromised patients should be 2-3 weeks. (Phin et al., 2014)

2.5 Amplification factor

2.5.1 Protozoa associations

Legionella can alive intracellular protozoan parasites (Kwaik et al., 1998) and the protected environment provided by the protozoan envelope reduces its susceptibility to disinfection and other harmful conditions. Legionella is residing within at least 20 species of amoebae, two species of ciliated protozoa and one species of slime mould (Table 3).

Table 3 Protozoan species found to harbour intracellular Legionella spp.

Type	References
Amoeba	
Acanthamoeba castellani	Rowbotham (1980)
Acanthamoeba culbertsoni	Fields et al. (1989)
Acanthamoeba hatchetti	Breiman et al. (1990b)
Acanthamoeba polyphaga	Rowbotham (1980, 1986)
Acanthamoeba palestinensis	Rowbotham (1986)
Acanthamoeba royreba	Tyndall and Domingue (1982)
Amoeba proteus strain x D	Park et al. (2004)
Comandonia operculata	Breiman et al. (1990b)
Echinamoeba exudans	Fields et al. (1989)
Filamoeba nolandi	Breiman et al. (1990b)
Hartmannella spp.	Fields et al. (1989)
Hartmannella cantabrigiensis	Rowbotham (1986); Breiman et al. (1990b)
Hartmannella vermiformis	Rowbotham (1986); Fields et al. (1989); Breiman et al. (1990b)
Naegleri fowleri	Newsome et al. (1985)
Naegleri gruberi	Rowbotham (1980)
Naegleri jadini	Rowbotham (1980)
Naegleri lovaniensis	Tyndall and Domingue (1982)
Paratetramitus jugosis	Breiman et al. (1990b)
Vahlkampfia spp.	Breiman et al. (1990b)

Table 3 (Continued) Protozoan species found to harbour intracellular Legionella spp.

Type	References
Vahlkampfia jugosa	Rowbotham (1986)
Vahlkampfia ustiana	Breiman et al. (1990b)
Ciliate	
Tetrahymena pyriformis	Fields et al. (1984)
Tetrahymena thermophile	Kikuhara et al. (1994)
Slime Mould	
Dictyostelium discoideum	Hagele et al. (2000)

Source: (Lau and Ashbolt, 2009)

2.5.2 Biofilm associations

Biofilms are defined as complex microbial communities featured by cells that are attached to a substratum and to each other by process of a matrix of self-produced extracellular polymeric substances (EPS) (Declerck, 2010). Biofilm formation can occur worldwide in natural and artificial environments, and on a range of different surfaces. Microorganisms, including L. pneumophila, form biofilms as a mechanism to withstand adverse conditions, such as low nutrients or temperature extremes. Surface adherence commonly occurs by process of an extracellular polysaccharide substance (EPS) secreted by the cells. This substance (the glycocalyx, or slime) is a hydrated polyanionic polysaccharide matrix produced by polymerases affixed to the lipopolysaccharide component of the cell wall (Bartram, 2007).

There are five recognized stages in the development of biofilm as follow:

1) Initial reversible attachment of free swimming microorganisms to surface

2) Permanent chemical attachment, single layer, bugs begin making slime

3) Early vertical development

4) Multiple towers with channels between maturing biofilm

5) Mature biofilm with seeding/dispersal of more free swimming microorganisms

The biofilms not only provide a source of nutrients for Legionella but also protect them from the antibiotics and other biocides. Biofilm prevention is an important role to control the proliferation of Legionella and is considered to be vital to control the Legionellosis.

2.5.3 Algal associations

Algae are the most abundant biofilm forming organisms on earth. On the surface water, algae can be both in planktonic form and biofilms. In biofilms, they may contain species which form toxins such as microcystin and represent a serious threat to human health (Wingender and Flemming, 2011). The Legionella have symbiotic relationship with some algae and Cyanobacteria, which may involve phosphorus metabolism and photosynthesis on the surface. Additionally, algal photosynthetic activity provides oxygen that can be used in aerobic respiration, which in turn produces CO_2, which may be available for algal photosynthesis. This mutualistic association between algae, Cyanobacteria and Legionella may occur in natural planktonic communities. The communities of cyanobacteria are Fischerella sp., Phornidium sp. and Oscillatoria sp. (Tison, Pope, Cherry and Fliermans, 1980). Recently, a highly sensitive amperometric immunosensor for microcystin detection in

algae and their biofilms has been reported. However, drinking water distribution systems and installations, algae do not occur due to lack of light (Wingender and Flemming, 2011). From the above data, the relationship of algae, cyanobacteria and Legionella may play an important role in the colonization and dispersal of Legionella in water systems.

2.6 Distribution of the Legionella spp. to humans

The transmission mode of Legionellosis is inhalation of Legionella organisms by contaminated water aerosols or occasionally via direct inoculation of Legionella into the wound. The transmission can occur from hospital potable hot water sources, potentially via shower aerosols (Hanrahan et al., 1987). The source of aerosol transmission of Legionella commonly found in cooling tower, shower, respiratory therapy device, swimming pool and fountain et cetera, which are shown in Table 4.

Table 4 Source of aerosol transmission of Legionella spp.

Source/reservoir	Likely mode of transmission
Taps	
Tap water	Direct wound contact—use of contaminated water to bathe patients
Showers	
Hot water supply	Inhalation of aerosols generated by shower nozzle
Baths	
Re-circulating hot water	Inhalation of aerosols generated by all-day-running-hot-water bath

Table 4 (Continue) Source of aerosol transmission of Legionella spp.

Source/reservoir	Likely mode of transmission
Water supply	Aspiration of contaminated water during delivery (birthing pool)
Respiratory equipment	
Re-usable oxygen humidifier	Inadequate cleaning/disinfection; inhalation of contaminated aerosols
Nebulizer	Malfunction of water distillation system; inhalation of contaminated aerosols
Room humidifier	Use of contaminated tap water to fill reservoir; inhalation of contaminated [cold mist] aerosol
Water features	
Decorative fountain	Stagnation of water during maintenance; inhalation of contaminated aerosol

Source: (Moore and Walker, 2014)

From the previous studies, In Italy (2014), they surveyed ten healthcare facilities to provide more information on the distribution of Legionella spp. by collected samples from air and water. They found 78.6% of L.pneumophila serogroup 6 (Lpn sg 6), 9.5% of Lpn sg 9, 5.5% of Lpn sg 1, 5.5% of Lpn sg 7 and 0.8% of Lpn sg 1and 12. These results showed that Lpn sg 6 was the serogroup, mostly found in water samples (Montagna et al., 2016).

In Canada, they collected 101 spa water samples and identified quantification of Legionella spp. by real-time PCR method compare with conventional culture

method. They found 13.86% (14 from 101) by culture method and 41.58% (42 from 101) by real-time PCR method. These two methods had low correlation. (Guillemet et al., 2010).

In Taiwan, they studied about distribution of Legionella in hot tub, spa and swimming pool by collected samples from 91 sites. They found Legionella in 21 sites (23%) and the most frequently detected was L.pneumophila. Moreover, they found Legionella in water temperature ranging from 22-50 °C and pH parameter found in range 5.0 to 9.0 (Hsu et al., 2006).

2.7 Distributions of Legionella spp. in Thailand

The Legionella spp. could be detected firstly in Thailand in 1984 and have been isolated in several regions of Thailand. The total number of cases during 1984-2002 were 17 patients and the most of Legionella species that caused the disease was Legionella pneumophila (Bovornkitti, 2010) .

Tishyadhigama et al. (1995) had surveyed for the contamination of Legionella in the environmental sources and cooling towers in several regions of Thailand. They found 57% of 94 cooling towers and 21.8% of 78 other environmental sources. The Legionella pneumophila serogroup 1 was the most of organisms predominating both in the cooling towers and other environmental sources.

Lertkhanawanichakul et al. (2004) had investigated Legionella spp. from the environments at Walailuk University in Nakronsrithammaraj provice. The samples were collected from the natural environmental air and man-made aquatic environments, including biofilm of potables. They found Legionella spp., 2 of 76 water samples (2.6%) from the environmental sources and 3 of 62 air samples (3.2%) but could not found Legionella spp. in the 30 biofilm samples. In addition, another

microorganisms (i.e. Acinetobacter, Pseudomonas, Staphylococcus and mold) were found in many samples. The exposure to high dose of microorganisms can lead to be the nosocomial infections in the immunocompromised patients.

Paveenkittiporn, Dejsirilert and Kalambaheti (2012) surveyed for Legionella organisms during 2003–2007 from various water resources from 33 provinces in Thailand. The samples were collected from cooling towers, storage tanks, chiller systems, hot springs, tap water, ponds, drinking-water containers and showers. The Legionella were firstly confirmed as Legionella species and identified as L. pneumophila based on PCR. The 256 isolates were confirmed as Legionella species. Among, 206 isolates (80.47%) were belonged to L. pneumophila and 50 isolates (19.53%) were identified as non-pneumophila when the samples were detected by DNA tree analysis.

Phares et al. (2007) studied the Legionella surveillance in 3489 patients with clinically-defined pneumonia in Sa Kaeo, the rural province in Thailand for 1 year. The samples were collected from sera, nasopharyngeal swabs, and urines for immunologic and molecular tests. Incidence of pneumonia was reported as a range from the lower limit to upper limit. The results showed that the incidence of pneumonia requiring hospitalization that was caused by Legionella longbeachae were 5–29 cases per 100,000 pneumonia patient population and no case of Legionella pneumophila pneumonia was observed. Other pathogenic microorganisms such as Mycoplasma pneumoniae and Chlamydia pneumoniae were frequently associated with severe pneumonia in Sa Kaeo too. But there were few patients who received antibiotics before collecting specimens, thus, these might cover atypical pathogens.

Buathong et al. (2013) had investigated Legionnaires' disease outbreak among EU travelers and hotel staffs in Phuket during 2006-2007. The information of each hotel guest was provided by home country officials for enquiring any symptoms after staying at the Phuket hotels. The water samples were collected from rooms and cooling towers in the hotel for Legionella cultures. Hotel staffs were tested for the Legionella pneumophila antibody by indirect fluorescent antibody (IFA) technique to identify the risk factors among hotel workers. The result showed that 5 confirmed cases (0.78%) and 1 presumptive case (0.16%) of Legionnaires' disease were traced from 645 Scandinavians staying at the hotels in Phuket. Among 118 hotel staffs, 78 cases (66.10%) had positive titer. The risk factors of Legionella infection were showers in the hotels which had Legionella and people aged more than 45 years old were group of increased risk for Legionella spp. infection.

In 2016, the Regional Medical Sciences Center 11/1, Phuket has been investigating the outbreak of Legionella spp. in Phuket, Phang-nga and Krabi provinces. The most common sources were water from showers, spas and faucets. They collected 1,508 water samples and found 116 samples positive for Legionella spp. but the amount of bacteria was not high enough to cause the disease in human. (Karnchanapimai et al., 2016)

2.8 Methods for Legionella detection

2.8.1 Cultural method

The Legionella detection method often uses the culture method which is the gold standard for the identification of Legionella spp. The first solid medium that is Mueller-Hinton agar supplemented with 1% IsoVitaleX and 1% hemoglobin (MHIH)

(Fields, Benson and Besser, 2002). Then L-cysteine hydrochloride can replace the IsoVitaleX reagent, and soluble ferric pyrophosphate can replace hemoglobin (Cordes et al., 1981). Later, starch is replaced with charcoal to detoxify the medium and the amino acid source is changed to be yeast extract, so a result is charcoal yeast extract agar (Feeley et al., 1979). The medium has been improved several times, until resulting in the medium currently used, buffered charcoal-yeast extract (BCYE) agar enriched with α - ketoglutarate (Edelstein, 1982). Legionella can be isolated from environmental water, water systems and specimens, including blood, lung tissue, lung biopsy specimens, respiratory secretions and stool. The antibiotic-containing media which perform better than the others for growing the stock strains and the clinical specimens contained with cefamandole, polymyxin B, anisomycin, organic buffer and α-ketoglutarate (Edelstein, 1981). For the water samples, BCYE agar containing glycine, vancomycin, polymyxin B and cycloheximide (GVPC) is a selective medium and suitable for Legionellae growing. The glycine, vancomycin and polymyxin B inhibit most non-target bacterial species, both gram-positive and gram-negative, including common contaminants such as Enterococci, Coliform, and Pseudomonas spp, while cycloheximide suppresses the growth of yeasts and moulds. These plates are incubated at 35 °C in a humid 2-5% CO_2 environment and examined after 4, 8 and 14 days of incubation (Leoni and Legnani, 2001).

The Legionella spp. generally produce small, blue-gray colonies, slow growing and have ground – glass appearance when examine with dissecting microscope. The suspected colonies are subcultured on BCYE agar, with and without cysteine. The Legionella can grow on BCYE with cysteine, but not grow on the BCYE without cysteine. The Legionella will be confirmed with biochemical test

(Hippurate hydrolysis) (Leoni and Legnani, 2001). The positive reaction performs a purple, a very light purple will be designated as weakly positive and shades of gray or a very light yellow will be reported as negative for hippurate hydrolysis.

2.8.2 Non-cultural methods

The several non-cultural methods have been developed to detect Legionella in environmental samples because the cultural method must wait for several days for growing Legionella. The non-cultural methods offer the potential of increased sensitivity and have a specificity more than the cultural method. However, the non-cultural methods have the disadvantage since they cannot provide the information regarding the viability of Legionella. The several non-cultural methods include, direct fluorescent antibody (DFA) staining, serological diagnosis (IFA and ELISA), urine antigen detection and detection of Legionella nucleic acid by polymerase chain reaction (PCR).

For the clinical and environmental samples, PCR has been successfully used to detect Legionella DNA and it is the rapid test for diagnosis of Legionellosis. There are several techniques available using rRNA (ribosomal RNA): 5S rRNA, 16S rRNA and mip gene (macrophage infectivity potentiator) used as target for PCR. Inoue, Takama, Yoshizaki and Agata (2015) had detected Legionella species in water samples and cooling tower water samples by using a combination of conventional plate culture, quantitative polymerase chain reaction (qPCR) and qPCR combined with ethidium monoazide treatment (EMA-qPCR) methods. The results showed that, EMA treatment decreased the number of Legionella-positive bath water samples detected by qPCR. In contrast, EMA treatment had no effect on cooling tower water

samples. So, EMA-qPCR is a useful method for the rapid detection of viable Legionella spp. from cooling tower water samples.

2.9 Legionella disinfection methods

The Legionella bacteria can cause Legionnaires' disease and Pontiac fever. This bacteria is commonly found in the natural water environment and water distribution systems. The water distribution systems have been reported that they are the sources of bacterial infections (Moore and Walker, 2014). So, the water systems need to get rid of bacteria. There are many disinfection methods involving thermal and chemical methods. For the disinfection of drinking water, chemical methods using disinfectants have been the most widely used (Kim, Anderson, Mueller, Gaines and Kendall, 2002).

Chemical methods

Chlorine is an oxidizing agent that efficiently uses as a disinfectant for controlling pathogens in domestic drinking water. The shock hyperchlorination is used to inactivate Legionella. Shock hyperchlorination is used by pulse injection of chlorine in water to achieve concentration of chlorine 20-50 ppm though out the system. After that water is drained and the system is mixed with water, the residual chlorine will return to normal concentration (0.5-1 ppm) (Lin, Stout, Yu and Vidic, 1998). When the shock hyperchlorination kills the Legionella bacteria in the water, then biofilm reduces dramatically. The performance of chlorine is more effective at higher temperature and higher pH.

Thermal methods

The thermal methods start with flushing all water outlets, faucets, and shower heads more than 30 min at >60 °C (140 °F) at distal outlets. At this temperature,

Legionella colonized in these sites are killed (Kim, Anderson, Mueller, Gaines and Kendall, 2002).

2.10 Microbiological evaluation of water sample quality

The microbiological parameters of water samples are compared with the standard of tap water recommended by Metropolitan Waterworks Authority, Thailand (based on WHO guideline 2011). The WHO's guideline has recommended the limitation of the water quality in microbiological parameters that tap water must not have any E.coli in 100 ml of water sample.

2.11 Research objectives

1. To detect Legionella spp. and other bacterial pathogens in water systems of nursing homes and spa pools in Bangkok and Nakhon Ratchasima provinces.

2. To prevent infections of Legionella and other bacterial pathogens in the elders in nursing homes and visitors who came to the swimming pools and spa, if microorganisms were found more than the accepted standards, these results were informed to the related persons to get rid of these microorganisms. After treatments, the samples at the infected sites were investigated again in order to eliminate the source of infections.

3. To determine the relationships between water parameters (temperature and pH value) and the prevalence of Legionella spp.

2.12 Research hypothesis

The detection of Legionella and other bacterial pathogens would be found in water systems of nursing homes and spa pools. After the suggestion and decontamination of Legionella, the samples sites that contaminated would be decreased.

CHAPTER III

MATERIALS AND METHODS

3.1 Preparation of the Legionella pneumophila bacteria, chemicals and reagents

Legionella pneumophila serogroup 1 ATCC 33152 were obtained from The Center of Scientific and Technological Equipment, Suranaree University of Technology. These Legionella pneumophila bacteria was used to be positive control. All chemicals and reagents used in this work were the laboratory grades or analytical grades, purchased from Himedia, Sigma-Aldrich and Amresco.

3.2 Instrumentation

Instruments for the detection of Legionella spp. in water samples from nursing homes and spa pools 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

Water samples were collected from the nursing homes and spa pools in Nakhon Ratchasima and Bangkok province, Thailand. Sixty samples were collected for detection of Legionella spp. and other bacterial pathogens, including viable heterotrophic bacteria, gram - negative bacteria, Staphylococcus spp. and Coliform that could cause the diseases. The water sample sites were shower heads, faucets and spa pools that could generate aerosol to the possibly exposed persons.

3.3.1 Shower heads and faucets

Water and biofilm samples from shower heads and sink faucets were collected by modified method of Cordes et al. (1981). The water samples were collected approximately 500 ml in the steriled containers.

3.3.2 Spa pools

Water samples from spa pools were collected approximately 500 ml in the sterile containers and stored samples at room temperature during transporting to the laboratory.

3.3.3 Samples processing

Each sample of water was collected in a sterile container which had 1 ml of a 10 mg/ml solution of sodium thiosulfate ($Na_2S_2O_3$) to neutralize residual disinfectants. The water temperature and pH value were determined immediately after collection (Nostro, Checchi, Ducci and Pesavento, 2011). The water was carried in the insulated containers at room temperature to the laboratory and processed within 24 h. The water samples were concentrated by filtration through 0.22 μm pore size cellulose acetate membrane filters (Millipore S.p.A., Milan, Italy) (Nostro, Checchi, Ducci and

Pesavento, 2011) and the membrane filters were cut into small pieces with aseptic technique, then put into a sterile tube that containing 1.5 ml sterile distilled water and vortexed for 30 seconds to remove bacterial cells from the membrane filters.

3.4 Microbiological analysis

3.4.1 Detection of Legionella species

The 1.5 ml of acid solution (HCl - KCl solution pH 2.2) were added to the concentrated water samples (from 3.3.3) for 5 minutes, then pipetted 1 ml to another tube that already contained 9 ml of sterile distilled water. The treatment water samples were tested by spread plate technique at undiluted and 10^{-1} dilution, 0.1 ml of each sample was placed in duplicate on Buffered Charcoal Yeast Extract (BCYE) agar and BCYE agar + glycine, vancomycin, polymyxin B and cycloheximide (GVPC) because no one medium will be optimal for the recovery of Legionella from every environmental site; so different selective media with various antibiotic combination in a BCYE were necessary. These plates were incubated at 37 °C in the humid chamber for 3-4 days. If there were Legionella bacteria, the blue-gray bacterial colonies would presence when using stereo microscope and ground – glass appearance when using dissecting microscope. The suspect colonies were cultured on BCYE and BCYE without L-cysteine for testing the requirement of cysteine by streak plate technique and incubated at 35 °C for 4 days. Legionella spp. were grown on BCYE but were not grown on BCYE without L-cysteine. L. pneumophila serogroup 1 ATCC 33152 were used as positive control. The biochemical tests were used to identifiy L. pneumophila from other legionellae by hippurate hydrolysis reaction (Hebert, 1981).

The suspect colony was selected from BCYE and emulsified in microcentrifuge tube containing 0.4 ml of 1% sodium hippurate. The suspension was placed in an incubator at 37 °C. After 18 to 20 h of incubation, 0.2 ml of the ninhydrin solution was added to each microcentrifuge tube. The contents were mixed by shaking and returned to the incubator for 10 min, then observed the color development within 20 minutes; all shades of purple will be read as a positive reaction, a very light purple was designated as weakly positive, and shades of gray or a very light yellow were reported as negative for hippurate hydrolysis. The number of typical colonies of Legionella spp. and L. pneumophila were counted, and reported as colony forming units per ml (CFU/ml).

3.4.2 Isolation and quantitation of total heterotrophic plate count

The determinations of heterotrophic bacteria were analyzed by 10-fold dilution series of the concentrate water sample. The 0.1 ml of concentrated water samples were cultured duplicate on plate count agar (PCA) with spread plate technique. All plates were incubated at 35 °C for 24 – 48 h (Reasoner, 2004). The number of colonies were counted and reported as colony forming unit per ml (CFU/ml).

3.4.3 Isolation of gram - negative bacteria

The gram – negative bacteria were cultured by spreading 0.1 ml of concentrated water samples on the Mac Conkey agar in duplicate. All plates were incubated at 35 °C for 24 h. The colonies of gram – negative were identified by morphology and biochemical tests (gram stain, oxidase test, catalase test, motility indole lysine test, OF-glucose test, simmons citrate agar and triple sugar iron agar,

which showed in Appendix D). The gram – negative bacteria were reported in genus by evaluated from Table 5.

3.4.4 Isolation of Staphylococcus spp.

The isolation of Staphylococcus spp. was analyzed by spread 0.1 ml of concentrated water samples on the selective medium, Manitol salt agar in duplicate. All plates were incubated at 35 °C for 24 h. The Staphylococcus aureus produced yellow colonies with yellow zones, there used for the selective isolation of presumptive pathogenic Staphylococcus species. The colonies of Staphylococcus spp. were confirmed by morphology and biochemical tests (gram stain and catalase test as shown in the Appendix D).

3.4.5 Isolation of Coliform and E.coli bacteria

The determination of Coliform bacteria was analyzed by inoculate sample water to lactose broth and incubated at 35 °C for 24-48 h. The positive tubes had a turbidity and produced gas within durham tube. The isolation of E.coli was analyzed by inoculated the solution in the positive tube to EC medium, streaked plate on eosin methylene blue agar (EMB), confirmed with urea test, gram stain and catalase test (Appendix D),then evaluated from Table 5.

Table 5 Biochemical test of gram-negative bacteria.

No	Organism	Lactose	Oxidase	Catalase	Motility	Indole	Urease	Triple sugar iron				Simmon citrate
								Butt	Slant	Gas	H_2S	
1	E.coli	+	-	+	+	+	-	Y	Y	+	-	-
2	Klebsella	+	-	+	-	+	+	Y	Y	+	-	+
3	Enterobacter spp.	+	-	+	+	-	-	Y	Y	+	-	+
4	Citrobacter	+	-	+	+	-	d	Y	Y/R	+	d	+
5	Salmonella Typhi	-	-	+	+	-	-	Y	R	-	+	-
6	Salmonella Parayphi-A	-	-	+	+	-	-	Y	R	+	-	-
7	S.typhi marium and other	-	-	+	+	-	-	Y	R	d	+	d
8	Shigella spp.	-	-	+	-	d	-	Y	R	-	d	-
9	Proteus	-	-	+	+	v	+	Y	R	+	+	d
10	Pseudomonas spp.	-	+	+	+	-	d	R	R	-	-	+
11	Vibrio cholerae	-	+	+	+	+	-	Y	Y	-	-	d
12	Paraheamolyticus	-	+	+	+	+	-	Y	Y	-	-	d
13	Serratia mercescus	-	-	+	d	-	d	Y	R	-	-	+
14	Yersina enterocolitire	-	-	+	+	d	+	Y	R	-	-	-
15	Providencia	-	-	+	+	+	-	Y	R	-	-	+

+ = Positive - = Negative Y = yellow R = red

v = variable (some strains positive, others strains negative)

d = result different in different species or strain

3.4.6 Repeated cultivation after decontamination of Legionella spp. at positive sites

The positive sites of Legionella spp. were reported to the nursing home and spa managers. The elimination of pathogens were done according to the recommended method of Bureau of food and water sanitation, Department of Health, Ministry of Public Health (Table 6). One month after elimination of pathogens, the repeated samples were collected and cultured again to prove that the tentative pathogenic microorganisms were destroyed completely.

3.4.7 The decontamination of Legionella spp.

The water disinfection recommended by Bureau of food and water sanitation, Department of Health, Ministry of Public Health.

Chemical methods

Chlorine powder is a white powder or white scales. The chlorine has to dissolve in the water and use the supernatant for disinfection.

- Prepare water into the glass or bucket, put the chlorine powder and mix with the water thoroughly until dissolve.
- Leave it until the undissolved powder precipitate.
- Add the supernatant into the jar or tank. Mix well. The amount and duration for elimination the pathogens was shown in Table 6.

Caution

- Keep out of reach of children. Store in a dry place and away from sunlight.
- Do not touch chlorine by hand.
- Do not eat directly.

Table 6 Amount and duration for elimination the pathogens.

Concentration of chlorine	Amount of chlorine powder	Water	Duration	Category of food
50 ppm	Half teaspoon	20 liter	30 min.	Vegetable, fruit
100 ppm	A teaspoon	20 liter	30 min.	Seafood
	A teaspoon	20 liter	2 min.	Container
	A teaspoon	20 liter	Cleaning	Building
2 ppm	A teaspoon	50 bucket	30 min.	Drinking water-water consumption
	1/8 teaspoon	8 bucket	30 min.	Drinking water-water consumption

3.5 Relationships between water parameters and the prevalence of Legionella pneumophila

The microbiological analysis was extended to the other informations by investigated the relationships between Legionella pneumophila and other water quality parameters (temperature and pH). The relationships were statistical analyzed by linear regression analysis (Leoni et al., 2005).

CHAPTER IV

RESULTS AND DISCUSSION

4.1 Survey and collection of samples

The water samples for the detection of Legionella spp. were collected from nursing homes and spa pools that could generate the aerosols with suspect of Legionellae contamination in droplets to the exposal persons. A total of 60 water samples sites were collected from showerheads, faucets and water tanks. The source of water samples were shown in Table 7.

Table 7 Source of samples for detection of Legionella spp.

Source	No. of samples
Nursing homes	
Bangkok	
- Showerheads	6
Nakhon Ratchasima	
- Faucets	2
- Showerheads	16
- Water tanks	6

Table 7 (Continued) Source of samples for detection of Legionella spp.

Source	No. of samples
Spa pools	
Nakhon Ratchasima	
- Faucets	30
Total	**60**

4.2 Detection of Legionella species

Thirty water samples collected from nursing homes and other 30 water samples from spa pools were examined for the detection of Legionella spp. by spread plate technique on BCYE agar and GVPC agar (BCYE agar with glycine, vancomycin, polymixin B and cycloheximide). Colonies of Legionella spp. on BCYE and GVPC appeared to be blue-gray with slightly convex, circular and total with a ground glass appearance (Figure 2). The suspected colonies were subsequently stained with gram stain and the result indicated that they were gram-negative, thin bacilli (Figure 3). Then, they were confirmed by sub–culturing on BCYE agar supplemented with and without L-cysteine. Legionella spp. are able to grow only on BCYE supplemented with L-cysteine. L. pneumophila was then distinguished from other Legionella spp. by hippurate hydrolysis reaction.

4.2.1 Nursing homes

A total of 30 water samples collected from nursing homes were examined for the Legionellae. The results demonstrated 5 positive out of 30 samples (16.67%) and were confirmed as L.pneumophila by hipurate hydrolysis reaction. These positive samples were collected from showers and water tanks; 2 samples from showers and 3 samples from water tanks. The mean value of L.pneumophila cell density was 13.20 CFU/100ml on GVPC agar, whilst no colony was seen on BCYE agar.

4.2.2 Spa pools

Thirty water samples collected from spa pools were screened for the Legionellae and detected for L.pneumophila by hipurate hydrolysis reaction. Thirteen samples (43.33%) were confirmed as L.pneumophila. These confirmed positive samples were collected from faucets of spa pools. The mean values of L.pneumophila density were 94.50 CFU/100ml on BCYE agar with cysteine and 435.92 CFU/100ml on GVPC agar, respectively. The L.pneumophila could grow well on GVPC agar which was the suitable agar medium for the growth of L.pneumophila because the antibiotics were added (Vancomycin, Polymyxin B and Cycloheximide) to inhibit gram-positive, fungal and yeast that could interrupt the growth of L.pneumophila.

The densities of the contaminated samples at the sampling sites were sumarized in Table 8.

Table 8 The density and interpretation level of the contaminated water samples from GVPC agar.

Samples	Density of L.pneumophila (CFU/100ml)
Nursing homes	
Showerhead N5 - Nursing homes No.3	24
Showerhead N13 - Nursing homes No.3	6
Water tank N27 - Nursing homes No.8	12
Water tank N28 - Nursing homes No.8	12
Water tank N29 - Nursing homes No.8	12
Spa pools	
Faucet S3 – spa pools No.3	30
Faucet S4 – spa pools No.4	54
Faucet S5 – spa pools No.4	6
Faucet S6 – spa pools No.4	12
Faucet S13 – spa pools No.6	42
Faucet S14 – spa pools No.6	492
Faucet S24 – spa pools No.7	759
Faucet S25 – spa pools No.7	2,469
Faucet S26 – spa pools No.7	126
Faucet S27 – spa pools No.7	528

Table 8 (Continued) The density and interpretation level of the contaminated water samples.

Samples	Density of L.pneumophila (CFU/100ml)
Spa pools	
Faucet S28 – spa pools No.8	72
Faucet S29 – spa pools No.8	21
Faucet S30 – spa pools No.8	1,056

The relative risk assessments of hazard levels of Legionella pneumophila

The relative risk assessments of hazard levels of Legionella pneumophila were categorized by Miller and Kenepp (1993) according to the density of L.pneumophila in water samples of cooling towers associated with outbreaks of Legionnaires'disease. The hazard levels of L.pneumophila were counted in CFU/100 ml. Our results of L.pneumophila densities in positive samples were categorized for the risk assessment and shown in Table 9 and Table 10.

Table 9 The relative risk assessment of Legionella pneumophila positive samples of nursing homes.

Density of Legionella pneumophila (CFU/100ml)	Risk category [a]	Amount of contaminated water samples
>100,000	Very high	-
10,000-99,999	High	-
1,000-9,999	Moderate	-
100-999	Low	-
<100	Very low	5

[a] The relative risk assessment according to Miller and Kenepp (1993)

This table showed that the densities of L.pneumophila in the all positive samples of nursing homes were in the very low category (<100CFU/100ml) and none were high or very high.

Table 10 The relative risk assessment of Legionella pneumophila positive samples of spa pools.

Density of Legionella pneumophila (CFU/100ml)	**Risk category** [a]	**Amount of contaminated water samples**
>100,000	Very high	-
10,000-99,999	High	-
1,000-9,999	Moderate	2
100-999	Low	4
<100	Very low	7

[a] The relative risk assessment according to Miller and Kenepp (1993)

This table showed that the densities of L.pneumophila in the positive samples of spa pools were mostly in the very low category (<100CFU/100ml), 2 of them were in the moderate risk category, 4 of them were low and none were high or very high.

The results showed that 18 of 60 total samples were positive (30%). The number of positive samples for Legionella spp. in this study was similar to other studies that were carried out in Thailand. The prevalence of Legionella spp. in water systems of hotels and resorts in the North-Eastern of Thailand was 24 from 75 (32.0%) hotels and resorts (A. Mahayotha, 2016). During 2003-2007, the prevalence of Legionella spp. in various water resources from 33 provinces in Thailand were investigated and 256 Legionella strains were isolated, among these, 206 isolates (80%) were belonged to L. pneumophila and 50 isolates were identified as non-pneumophila by DNA tree analysis. (Paveenkittiporn, 2012). In 2004, Borella et al. studied Legionella infection risk from domestic hot water and found that 22.6% (33/146) were Legionella spp. and 38.4% (56/146) were Pseudomonas spp.

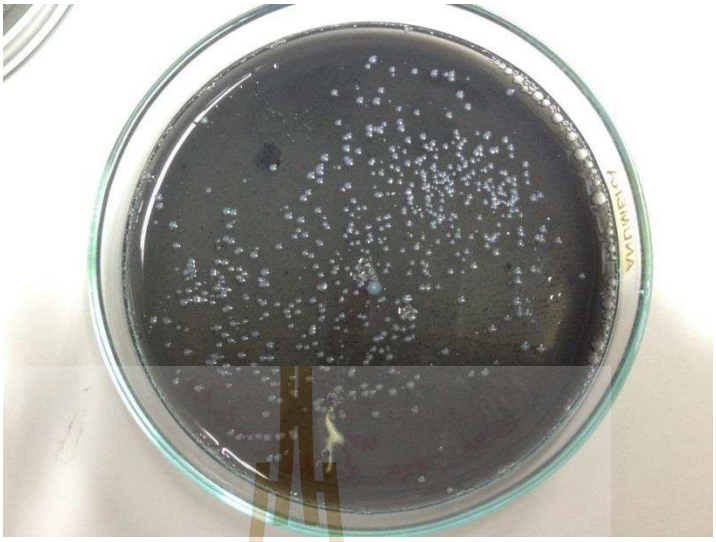

Figure 2 Legionella spp. colonies grow on GVPC agar.

The characteristic of Legionella colonies were the blue-gray bacterial colonies, glistening, convex, and circular with an entire edge.

Figure 3 Gram stain of Legionella spp. under compound light microscope (1000x magnification).

4.3 Detection of other microorganisms

This research collected 60 samples in total from nursing homes and spa pools. All samples were cultivated to screen for Legionella spp. and other microorganisms including total heterotrophic bacteria, gram-negative bacteria, Staphylococcus spp. and total Coliform bacteria.

The densities of heterotrophic bacteria contaminated in water samples collected from nursing homes and spa pools are illustrated in Table 8. The average mean of heterotrophic bacteria contained in water samples collected from nursing homes was 1.05×10^3 CFU/ml with ranged from 1.20 to 1.62×10^4 CFU/ml. For spa pools, the average mean was 8.61×10^2 CFU/ml with ranged from 6.00 to 1.22×10^4 CFU/ml.

Not only heterotrophic bacteria but there were other microorganisms contaminated in water samples. These microorganisms were found in water samples from both nursing homes and spa pools; Coliform 16.67% (10 from 60 samples), E.coli 5% (3 from 60 samples), Staphylococcus spp. 16.67% (10 from 60 samples) and some gram-negative bacteria including Pseudomonas spp. (35.00%), Enterobacter spp. (8.33%), Citrobacter spp. (5.00%) and Acinetobacter spp. (8.33%). The various microorganisms in each sample site were demonstrated in Table 11 and Table 12.

Table 11 The microorganisms in the water samples of nursing homes.

Source	Total plate count (CFU/ml)	Legionella spp. (CFU/100 ml)	Gram-negative	Staphylococcus spp.	Coliform	E.coli
N1	32.20	-	E.coli, Enterobacter spp.	✓	✓	✓
N2	39.50	-	-	✓	-	-
N3	6.40	-	-	-	-	-
N4	6.48×10^2	-	Pseudomonas spp.	✓	-	-
N5	4.26×10^3	24.00	Pseudomonas spp.	✓	-	-
N6	1.96×10^2	-	E.coli, Acinetobacter spp.	✓	✓	✓
N7	1.48×10^2	-	Enterobacter spp., Acinetobacter spp.	-	✓	-
N8	11.70	-	Enterobacter spp.	-	✓	-
N9	1.16×10^3	-	Acinetobacter spp., Pseudomonas spp.	-	-	-
N10	4.16×10^2	-	Pseudomonas spp.	-	-	-
N11	4.64×10^3	-	Pseudomonas spp.	✓	-	-
N12	1.76×10^2	-	-	-	-	-
N13	37.90	6.00	-	-	-	-
N14	38.30	-	Acinetobacter spp.	-	-	-

Table 11 (Continued) The microorganisms in the water samples of nursing homes.

Source	Total plate count (CFU/ml)	Legionella spp. (CFU/100 ml)	Gram-negative	Staphylococcus spp.	Coliform	E.coli
N15	37.50	-	-	-	-	-
N16	53.80	-	Pseudomonas spp.	-	-	-
N17	4.11×10^{2}	-	-	-	-	-
N18	1.62×10^{4}	-	-	-	-	-
N19	2.53×10^{2}	-	Pseudomonas spp.	-	-	-
N20	2.72×10^{3}	-	-	✓	-	-
N21	1.20	-	-	✓	-	-
N22	26.50	-	-	-	-	-
N23	2.40	-	-	-	-	-
N24	1.40	-	Acinetobacter spp.	-	-	-
N25	22.00	-	-	-	-	-
N26	1.80	-	Pseudomonas spp.	-	-	-
N27	7.70	12.00	E.coli	-	✓	✓
N28	18.20	12.00	Enterobacter spp.	✓	✓	-
N29	2.30	12.00	Pseudomonas spp.	-	-	-
N30	1.50	-	Pseudomonas spp.	-	-	-

Table 12 The microorganisms in the water samples of spa pools.

Source	Total plate count (CFU/ml)	Legionella spp. (CFU/100 ml)	Gram-negative	Staphylococcus spp.	Coliform	E.coli
Spa pools						
S1	22.60	-	Pseudomonas spp.	-	-	-
S2	1.93×10^{2}	-	-	-	-	-
S3	1.29×10^{2}	30.00	Citrobacter spp.	-	✓	-
S4	2.94×10^{2}	54.00	Pseudomonas spp.	-	-	-
S5	39.10	6.00	-	-	-	-
S6	28.50	12.00	-	-	-	-
S7	13.60	-	Pseudomonas spp.	-	-	-
S8	1.22×10^{4}	-	Pseudomonas spp.	-	-	-
S9	2.98×10^{2}	-	Pseudomonas spp.	-	-	-
S10	1.84×10^{3}	-	Pseudomonas spp.	-	-	-
S11	32.70	-	Pseudomonas spp.	-	-	-
S12	40.80	-	-	-	-	-
S13	2.00×10^{2}	42.00	-	✓	-	-
S14	1.50×10^{2}	4.92×10^{2}	-	-	-	-

Table 12 (Continued) The microorganisms in the water samples of spa pools.

Source	Total plate count (CFU/ml)	Legionella spp. (CFU/100 ml)	Gram-negative	Staphylococcus spp.	Coliform	E.coli
S15	52.40	-	-	-	-	-
S16	44.20	-	-	-	-	-
S17	1.09×10^{2}	-	-	-	-	-
S18	5.70×10^{3}	-	-	-	-	-
S19	3.92×10^{2}	-	-	-	-	-
S20	2.23×10^{2}	-	-	-	-	-
S21	5.40×10^{2}	-	-	-	-	-
S22	31.60	-	Pseudomonas spp.	-	-	-
S23	6.00	-	Pseudomonas spp. Citrobacter spp.	-	- ✓	-
S24	53.00	7.59×10^{2}	-	-	-	-
S25	2.18×10^{2}	2.47×10^{3}	-	-	-	-
S26	1.84×10^{2}	1.26×10^{2}	-	-	-	-
S27	2.82×10^{2}	5.28×10^{2}	-	-	-	-

Table 12 (Continued) The microorganisms in the water samples of spa pools.

Source	Total plate count (CFU/ml)	Legionella spp. (CFU/100 ml)	Gram-negative	Staphylococcus spp.	Coliform	E.coli
S28	2.52×10^{2}	72.00	Citrobacter spp.	-	✓	-
S29	1.92×10^{3}	21.00	Pseudomonas spp.	-	-	-
S30	3.42×10^{2}	1.06×10^{3}	Pseudomonas spp.	-	-	-
			Enterobacter spp.		✓	

✓ = Found

- = Not found

Regarding to Table 11 and Table 12, the total plate count or heterotrophic plate counts (HPC) is a method used to measure variety of bacteria that are common in water for quality assessment of drinking water in storage tanks and in water distribution systems. Hetero-trophic plate counts are not the indicators of pathogenic conditions but some of them such as Pseudomonas spp. is the opportunistic pathogens that can cause some infections in skin and lung and also the other, Aeromonas spp. cause gastroenteritis (Amanidaz, Zafarzadeh and Mahvi, 2015). Therefore, if heterotrophic plate counts are high then the risk are increase too. The National primary drinking water regulations of Environmental Protection Agency, United States of America recommended that heterotrophic plate counts should no more than 500 CFU/ml for safety water systems. Moreover, the Coliform bacteria were found in water samples collected from nursing homes and spa pools. The Coliform bacteria included Citrobacter spp., Enterobacter spp., Hafnia spp., Klebsiella spp. and E.coli. They were found in intestines of human and warm-blooded animals, so they were used as indicator for fecal contamination. The Enterobacter, Acinetobacter, Citrobacter and E.coli were found to be 8.33%, 8.33%, 5% and 5%, respectively. The Enterobacter, Citrobacter and E.coli could cause many diseases including septicemia, pneumonia, meningitis and urinary tract infections. While the Acinetobacter could cause a variety of diseases, ranging from pneumonia to serious blood or wound infections. Therefore, the Queensland health swimming and spa pool water quality and operational guidelines 2004 recommended the microbiological criteria that thermo tolerant (fecal) Coliform or E.coli should not be detected in 100ml and also Pseudomonas aeroginosa too, for reduce the risk contamination and potential for

illness. The Pseudomonas spp. were found to be the most common gram-negative bacteria contaminated in water samples because Pseudomonas spp. were mostly resistant to antibiotics and secreted extracellular enzyme, toxin and had ability to develop biofilm on many surfaces, so the infections of Pseudomonas spp. might be difficult to eradicate. They were found 35% (21 from 60 samples) and most of them were found in showerheads and faucets from nursing homes.

These microorganisms found in this present study were similar to other studies. The study of water system in ICU wards, hospitals in Tehran of Iran was indicated that the Legionella pneumophila, Pseudomonas aeruginosa and Acinetobacter were found 9.6%, 11.4% and 1.8%, respectively. The Legionella pneumophila, Pseudomonas aeruginosa, and Acinetobacter baumanii could survive in water released from their biofilm into the water stream. These posed a high risk of infection to people. Legionellosis and other nosocomial waterborne infections were occurred by the microorganisms presented and amplified in water reservoir, associated with water biofilms, and the transmission of bacteria (aerosolization, ingestion, and contact) (Yaslianifard, 2012).

In this study, the number of spa pools that found Legionella spp. was higher than nursing homes since the water systems of spa pools were high temperature which was an ideal temperature for Legionella spp. growing. Moreover, the usability of water systems in spa pools might not be opened every day, thus, bacteria at the faucets might accumulate and grow while the water systems of the nursing homes normally were opened every day. Therefore, the spa pools had chances to find Legionella spp. more than the nursing homes.

In this study, Staphylococcus spp. were found 16.67% (10 from 60 samples). Comparing to Lechevallier and Seidler (1980), they found S.aureus 6.25% (20 from 320 samples) in rural drinking water. The Staphylococcus spp. is a gram-positive bacteria that can cause a variety of diseases in human such as skin abscesses, pustules, septicemia, enterocolitis, osteomylitis, and pneumonitis and is an agent of food poisoning because they can produced endotoxin into the food that cause vomiting and diarrhea (Lechevallier and Seidler, 1980).

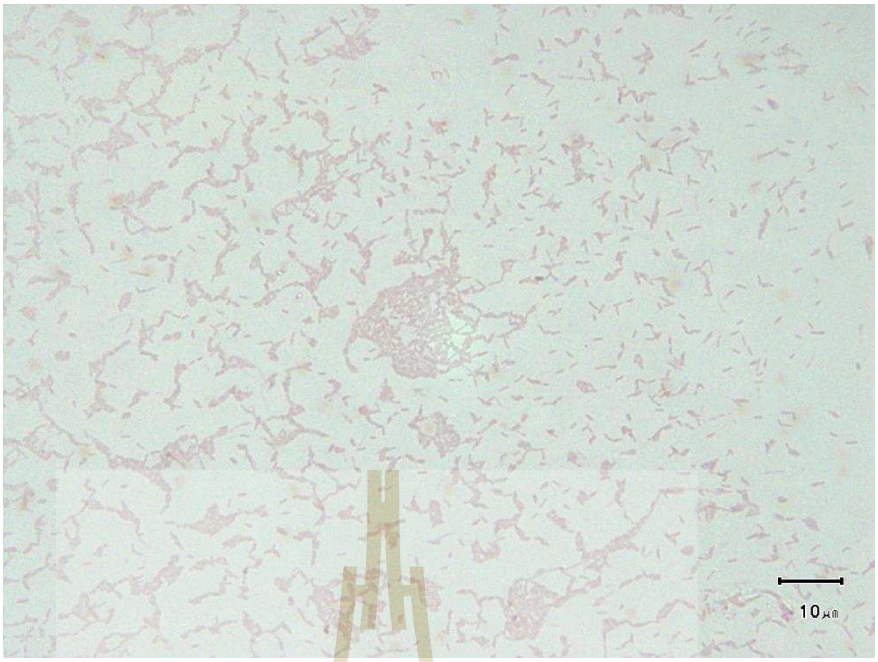

Figure 4 Gram stain of Citrobacter spp. under compound light microscope (1000x magnification).

Figure 5 Gram stain of Pseudomonas spp. under compound light microscope (1000x magnification).

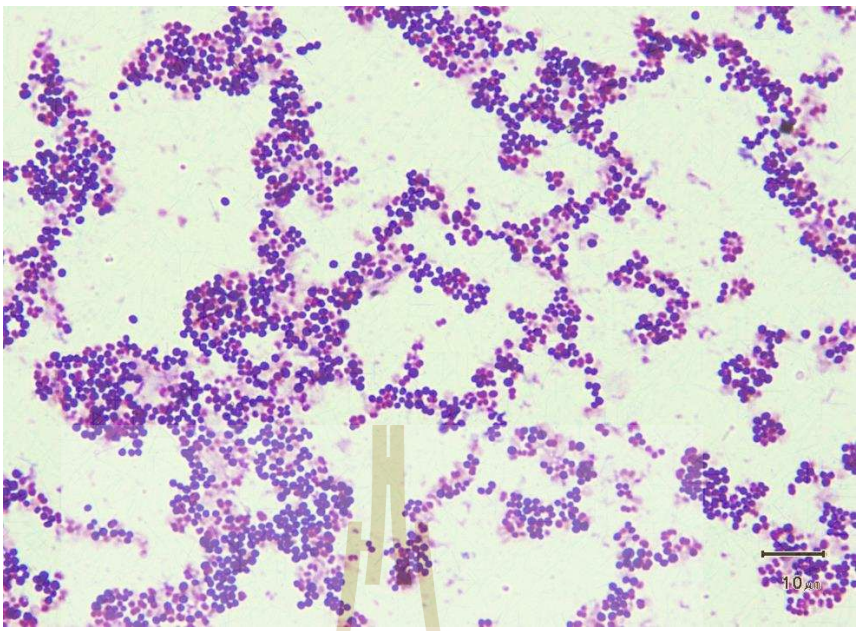

Figure 6 Gram stain of Staphylococcus spp. under compound light microscope (1000x magnification).

Figure 7 Gram stain of Acinetobacter spp. under compound light microscope (1000x magnification).

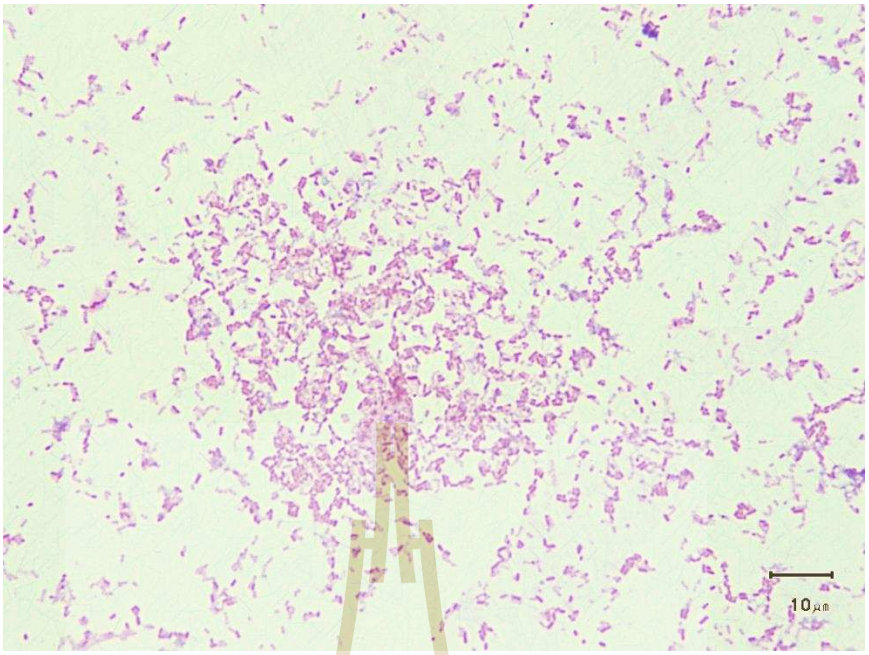

Figure 8 Gram stain of Enterobacter spp. under compound light microscope (1000x magnification).

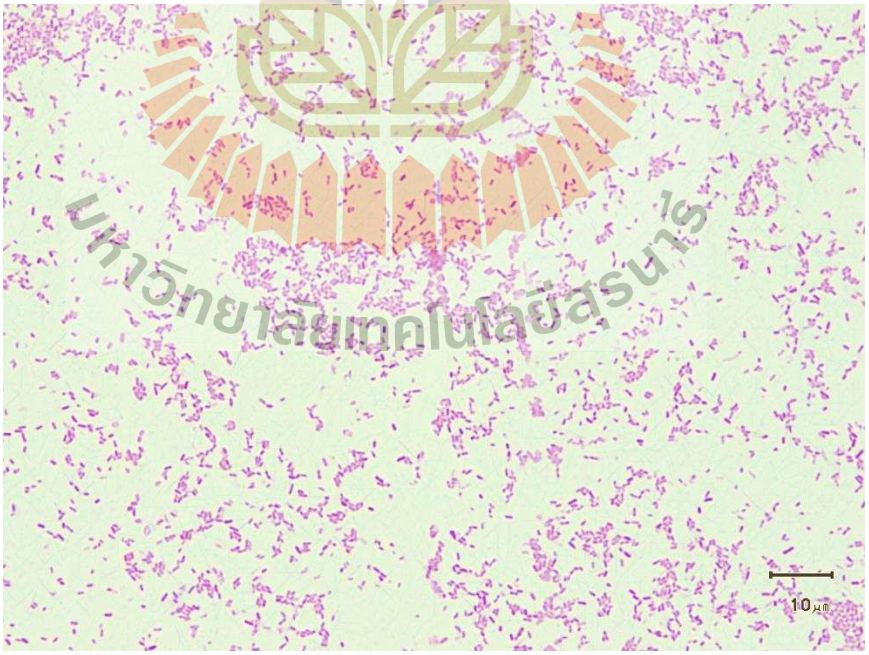

Figure 9 Gram stain of E.coli spp. under compound light microscope (1000x magnification).

4.4 Microbiological quality of water samples

The microbiological parameters of tap water used in this research were based on the guideline recommended by Metropolitan Waterworks Authority, Thailand (based on WHO's guideline 2011) (Appendix C). The WHO's guideline recommended that microbiological parameters of the good water quality of tap water must not have any E.coli in 100 ml of water sample and no contamination of Legionella spp. recommended by National primary drinking water regulations of Environmental Protection Agency, United States of America for safety water in nursing homes and spa pools.

The results showed that 3 from 60 tap water samples were E.coli contaminations and 18 from 60 tap water samples contaminated with Legionella spp. Thus, the water quality of these particular samples did not meet the criteria of safety water according to the WHO's guideline. However, the water samples that contaminated E.coli, we were reported the results of water analysis and provided the suggestion about elimination of contaminated samples. While, the water samples that contaminated of Legionella spp., we were reported and elimination of contaminated samples then recollected the water samples for analysis to prove that the water samples were not contaminated of the Legionella spp.

4.5 Physical analysis of samples

The water samples from nursing homes and spa pools were evaluated for pH and temperature. The results of the water parameters were concluded in Table 13 and Table 14.

Table 13 Physical parameters of water samples collected from nursing homes (N=30).

Parameters	Source	Mean±SEM	Median	Range (min-max)
pH	Faucets	7.36±0.29	7.36	7.07-7.64
	Showerheads	7.30±0.07	7.37	6.71-7.75
	Water tanks	7.36±0.26	7.54	6.43-8.09
Temperature (°C)	Faucets	28.25±1.75	28.25	26.50-30.00
	Showerheads	29.14±0.53	29.50	25.00-35.00
	Water tanks	25.08±1.19	24.50	22.50-30.00

The average pH values of water samples from faucets, showerheads and water tanks of nursing homes were 7.36±0.29, 7.30±0.07and 7.36±0.26, respectively. The pH of water was in normal range which was 6.5 to 8.5, recommended by Metropolitan Waterworks Authority, Thailand (based on WHO's guideline 2011). For water temperature, the average value of water samples from faucets, showerheads and water tanks of nursing homes were 28.25±1.75 °C, 29.14±0.53 °C and 25.08±1.19 °C, respectively and the optimal water temperature was 35 °C (Tison, 1980).

Table 14 Physical parameters of water samples from spa pools (N=30).

Parameters	Source	Mean±SEM	Median	Range (min-max)
pH	Faucets	7.61±0.08	7.66	6.55-8.29
Temperature (°C)	Faucets	27.09±0.50	27.50	21.50-33.50

From the study, the pH range of water samples from faucets of spa pools was 6.55 to 8.29. The average value was 7.61±0.08. The result indicated that the pH value was in normal range 7.2 to 7.8. (Queensland health swimming and spa pool water quality and operational guidelines, 2004). The water temperature of spa pools ranged from 21.50 to 33.50 °C with the average value 27.09±0.50 °C. The water temperature enhanced the bacterial growth at exceeding 26 °C. The optimum temperature is approximately 38 °C. (Queensland health swimming and spa pool water quality and operational guidelines, 2004). These temperature and pH were suitable for the growth of several bacteria.

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

The relationships between outbreak of Legionella and physical parameters and bacteriological parameters of all water samples were examined by Pearson' s correlation analysis (IBM SPSS Statistics version 23).

Table 15 Statistical analysis of Pearson's correlation analysis between water quality parameters (temperature and pH) and the outbreak of Legionella spp.

Parameter	Source	r	Sig.	p
Nursing homes				
pH	Faucets	-	-	0.05
	Showerheads	-0.399	0.066	0.05
	Water tanks	0.790	0.061	0.05
Temperature	Faucets	-	-	0.05
	Showerheads	-0.103	0.647	0.05
	Water tanks	-0.597	0.211	0.05
Spa pools				
pH	Faucets	0.331	0.074	0.05
Temperature	Faucets	0.111	0.561	0.05

The results from statistical analysis of the relationship between water parameters (pH and temperature) and the prevalence of Legionella spp. showed that no statistical significant correlation in both nursing homes and also in the spa pools because Sig.>0.05 and r value (Pearson correlation) were very low. The correlation coefficient ranged between −1 to 1 if the value was zero indicated that there was no correlation between the two variables. So, these results were suggested that the relationship between water parameters (pH and temperature) and prevalence of Legionella spp. had no statistical significant correlation in nursing homes and also in the spa pools.

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

The positive sites of Legionella spp. were reported to the nursing homes and spa managers for decontamination of microorganism's contaminants according to the recommendation of Bureau of food and water sanitation, Department of Health, Ministry of Public Health. After one month of decontamination, water samples were recollected and cultured for microorganism's contamination again to prove that whether the Legionella was destroyed completely or not.

After decontamination, Legionella was mostly eliminated except in some samples of spa pools, Legionella still remained but the density were decreased. The other microorganisms (heterotrophic bacteria and gram-negative bacteria) still remain in those sites. Heterotrophic bacteria were decreased in some samples of nursing homes and spa pools. The Staphylococcus spp. was not found. These results were shown in Table 16 and 17.

Table 16 Legionella spp. and other microorganisms detected from water samples before and after elimination in positive sample sites of nursing homes.

Sample no.	Density of Legionella spp. (CFU/100ml)		Total plate count (CFU/ml)		Other microorganisms	
	before	after	before	after	before	after
Showerhead N5	24	0	4,260	46.20	Pseudomonas spp.	Pseudomonas spp.
Showerhead N13	6	0	37.90	2.86	Not found	Not found
Water tank N27	12	0	7.70	11.90	E.coli	E.coli
Water tank N28	12	0	18.20	6.10	Enterobacter spp.	Enterobacter spp.
Water tank N29	12	0	2.30	6.80	Pseudomonas spp.	Pseudomonas spp.

Table 17 Legionella spp. and other microorganisms detected from water samples before and after elimination in positive sample sites of spa pools.

Sample no.	Density of Legionella spp. (CFU/100ml)		Total plate count (CFU/ml)		Other microorganisms	
	before	after	before	after	before	after
Faucet S3	30	1,311	129	465	Citrobacter spp.	Not found
Faucet S4	54	204	294	37.62	Pseudomonas spp.	Not found
Faucet S5	6	30	39.1	33.81	Not found	Not found
Faucet S6	12	24	28.5	200	Not found	Pseudomonas spp.
Faucet S13	42	0	200	10.6	Not found	Not found
Faucet S14	492	0	150	44.64	Not found	Not found
Faucet S24	759	6	53	9.6	Not found	Pseudomonas spp.
Faucet S25	2,469	6	218	4.5	Not found	Not found
Faucet S26	126	0	184	33.3	Not found	Not found

Table 17 (Continued) Legionella spp. and other microorganisms detected from water samples before and after elimination in positive sample sites of spa pools.

Sample no.	Density of Legionella spp. (CFU/100ml)		Total plate count (CFU/ml)		Other microorganisms	
	before	after	before	after	before	after
Faucet S27	528	6	282	428	Not found	Not found
Faucet S28	72	0	252	220	Citrobacter spp.	Not found
Faucet S29	21	0	1,920	199.04	Pseudomonas spp.	Pseudomonas spp.
Faucet S30	1,056	0	342	262	Enterobacter spp., Pseudomonas spp.	Not found

Regarding to the water quality of water samples collected from nursing homes, the positive sites may be risk to the elder person that stayed there. However, the people age over 60 years included smokers have more risk than the other people because they have low immunity and the Legionella infection was found in male more than female. (Elverdal et al., 2013) The L.pneumophila positive sites (showerhead and water tank) were reported to manager of nursing homes for elimination of the microorganisms and they well-cooperated. After elimination, the water samples were recollected and cultivated to screen for microorganisms contamination. The total bacteria counts of microorganisms at the positive sites were decreased in some water samples and absence of Legionella. In the spa pools, the positive sites may be risk to the customers and spa pool keepers. The route of infection can transmit by inhalation of bacteria contaminated as aerosol forms. The L.pneumophila positive sites (faucet from spa pools) were reported and advised the spa managers about the risk of infection and how to decontaminate the microorganisms. After decontamination, the water samples were collected again and repeated the cultivation. The total bacteria counts of microorganisms and Legionella mostly were decreased. For other Legionella sample sites that were remain positive, after repeat decontamination again, the water samples will be recollected and cultivated later.

Therefore, the findings of this study should be concerned by the epidemiologists since Legionella spp. and other bacterial pathogens in nursing home where there have a lot of vulnerable people and in spa pool may cause the outbreak of Legionellosis and other infections caused by poor quality of the water systems.

CHAPTER V

CONCLUSION

The previous epidemiological studies of Legionella spp. reported that Legionella spp. were considered to be the major cause of Legionnaires' disease (LD) in the water systems of large buildings including hospitals, nursing homes and hotels. Legionella is a common cause of hospital-acquired pneumonia, especially in immune-compromised patients (Yu et al., 2008). Legionella spp. are gram-negative and non-spore-forming bacteria. The representative species of the genus is Legionella pneumophila that can cause the Legionellosis. The Legionellosis is a respiratory disease that can be divided into two clinical identities, Pontiac fever and Legionnaires' disease. The symptoms of Pontiac fever are similar to a mild case of the flu, but the Legionnaires' disease presents more severe symptoms including pneumonia. The Legionella spp. are commonly found in natural water environments (e.g., rivers, lakes, lagoon and reservoirs) and human-made water systems (e.g., cooling tower, water heater tanks, fountain, humidifiers and spa pools). The most common mode of transmission of Legionella spp. is inhalation of contaminated aerosols. In this study, the water samples collected from 30 nursing homes and 30 spa pools in Bangkok and Nakhon Ratchasima provinces were examined for the presence of Legionella pneumophila and other bacterial pathogens by culture and biochemical methods.

The Legionella spp. were detected in some water systems of nursing homes and spa pools; 5 from 30 (16.67%) water samples collected from nursing homes and 13 from 30 (43.33%) were detected from spa pools. The number of positive samples for Legionella spp. in this study was similarly to other previous studies in Thailand. The prevalence of Legionella spp. in water systems of hotels and resorts in the North-Eastern of Thailand was found 24 from 75 (32%) hotels and resorts. Similarly, the hot water recirculation systems in hotels and nursing homes at Spain were analyzed for Legionella spp. The Legionella pneumophila sg.1 was found 50 from 231 (22%). The elderly, smoker people and the immunosuppressed patients including the managers or staffs of nursing homes and spa pools are considered to be high risk for this particular infection. Additionally, routine laboratories in Thailand do not screen for Legionella spp., thus when the patients are infected with Legionella spp., they were overall diagnosed as pneumonia. Moreover the temperature in Thailand is a suitable range for Legionella growth. So, the Legionella positive sites (faucets, showerheads and water tanks) were reported to the managers of nursing homes and spa pools, discussed about public health problems and how to eliminate the microorganisms. After decontamination at the Legionella positive sites, water samples were recollected and cultivated for Legionella and other microorganisms. The results showed that Legionella and other microorganisms reduced from the first collection.

For the Legionella sample sites that still remained positive, we reported to the managers and told them that the water systems should be decontaminated again. The water samples were recollected after cleaning and elimination of the microorganisms. The decontamination of the sample positive sites were done four times to get rid of

the microorganisms as much as possible. The water samples were still positive but tend to decrease. We suggested more about elimination of microorganisms and told them to send the water samples again until there is no contamination to be safe for customers.

The gram-negative bacteria that were found in both nursing homes and spa pools were Pseudomonas spp. (35%), Enterobacter spp. (8.33%), Acinetobacter spp. (8.33%), and Citrobacter spp. (5%). The Staphylococcus spp. was accounted for 16.67%. The Coliform bacteria and E.coli were found 16.67% and 5%, respectively. The E.coli was found only in the nursing homes. After decontamination at the positive sites, samples from decontaminated sites were re-evaluated. The other microorganisms decreased, except Pseudomonas spp., E. coli and Enterobacter spp. These microorganisms are resistant to various disinfectants and commonly found in the environment, so they can still be found in water samples. Thus, these microorganisms are still at risk for the people especially the elderly and immunosuppressed patients by they can cause infection via wound, eyes, ears, skin and soft tissue, so the people should avoid the chance of infection. The average pH and temperature of this study were in standard value according to Queensland health swimming and spa pool water quality and operational guidelines 2004. The relationship between water parameters (pH and temperature) and the prevalence of Legionella spp. had no statistical significant correlation in both nursing homes and spa pools.

Therefore, this study showed the prevalence of Legionella spp. and other bacterial pathogens which possibly cause infection in both nursing homes and spa pools in Bangkok and Nakhon Ratchasima provinces. Moreover, the studies have motivated the intendants to aware the danger of Legionella spp. and concern about the possible outbreak of Legionella spp. and other bacterial infections.

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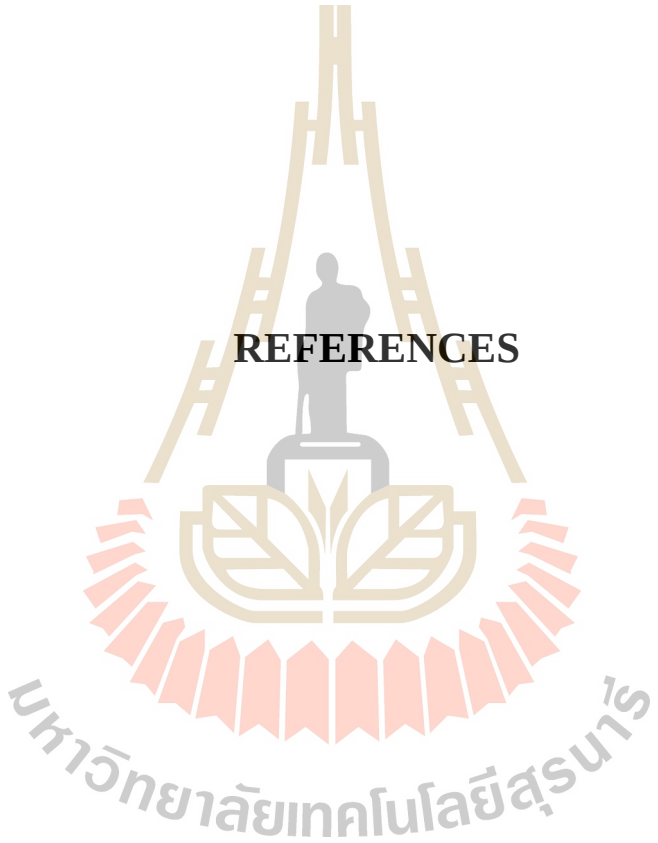

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APPENDICES

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.H2O	0.4 g
Agar	15.0 g
Final pH 6.9 (± 0.2)	

Preparation of medium: dissolved charcoal, yeast extract, ACES buffer, alphaketoglutarate 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 Lcysteine 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 filtersterilized antibiotics and mix. The medium was dispensed into sterilized petri dishes.

3. 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.

4. 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.

5. Eosin methylene blue agar (EMB agar)

Peptic digest of animal tissue	10.0 g
Dipotassium phosphate	2.0 g
Lactose	10.0 g
Eosin - Y	0.4 g
Methylene blue	0.065 g
Agar	15.0 g

Final pH (at 25 °C) 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 heated until dissolved. The medium was autoclaved at 121 °C for 15 min. Dispensed into sterilize petri dishes.

6. Mannitol Salt Agar (MSA agar)

Proteose peptone	10.0 g
Beef extract	1.0 g
Sodium chloride	75.0 g
D-Mannitol	10.0 g
Phenol red	0.025 g
Agar	15.0 g

Final pH (at 25 °C) 7.4±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 petridishes.

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 GUIDELINE 2011)

Table 1 C Standard of tap water recommended by Metropolitan Waterworks Authority (based on WHO guideline 2011).

Parameters	Units	Recommend
1. Bacteriology quality		
E. coli None	Not found/100 ml	Not found/100 ml
2. Physical and chemical quality		
Appearance color	True color unit	15
Turbidity	NTU	5
Taste and odor	-	-
pH	-	6.5-8.5
Arsenic	mg/l	0.01
Cadmium	mg/l	0.003
Chromium	mg/l	0.05
Cyanide	mg/l	0.5

Table 1 C (Continued).

Parameters	Units	Recommend
Lead	mg/l	0.01
Inorganic Mercury	mg/l	0.006
Selenium	mg/l	0.04
Fluoride	mg/l	0.7
Chloride	mg/l	250
Copper	mg/l	2
Iron	mg/l	0.3
Manganese	mg/l	0.1
Aluminium	mg/l	0.9
Sodium	mg/l	200
Sulfate	mg/l	250
Zinc	mg/l	3
Total dissolved solids	mg/l	1,000
Nitrate as NO_3^-	mg/l	50
Nitrite as NO_2^-	mg/l	3
Trichloroethene	mg/l	0.02
Tetrachloroethene	mg/l	0.04
Microcystin-LR	mg/l	0.001
3. Pesticides		
Aldrin/Dieldrin	µg/l	0.03

Table 1 C (Continued).

Parameters	Units	Recommend
Chlordane	µg/l	0.2
DDT and metabolites	µg/l	1
2,4-D	µg/l	30
Heptachlor and Heptachlor epoxide	µg/l	0.03
Hexachlorobenzene	µg/l	1
Lindane	µg/l	2
Methoxychlor	µg/l	20
Pentachlorophenol	µg/l	9
4. Trihalomethanes sum of the ratio		1
Chloroform , $CHCl_3$	mg/l	0.3
Bromodichloromethane , $CHBrCl_2$	mg/l	0.06
Dibromochloromethane , $CHBr_2Cl$	mg/l	0.1
Bromoform , $CHBr_3$	mg/l	0.1
5. Radioactive		
Gross alpha activity	bq/l	0.5
Gross beta activity	bq/l	1

APPENDIX D

BIOCHEMICAL TEST OF BACTERIA

Gram stain – Used the sterile cooled loop to place a drop of sterile water or saline solution on the slide. The loop was sterilized again and picked up a small sample of a bacterial colony and stirred into the drop of water on the slide and smeared to thin layer. The smeared slide was heat fixed to adhere the bacteria to the slide. Then, the slide was flooded with crystal violet for 1 minute and rinsed with tap water or distilled water. Followed by Gram's Iodine solution for 1 minute, rinsed with tap water or distilled water again. The slide was decolorized by 95% ethyl alcohol for 10 seconds, washed off with tap water. Finally, the slide was flooded with safranin to counter-stain for 1 minute and rinsed with tap water, the dried slide was viewed under light microscope with oil-immersion.

Catalase test – Used the sterile cooled loop to collect a small amount of bacteria from 18- 24 h. colony and placed it onto the slide. Then used the dropper or Pasteur pipette to place a drop of 3% H_2O_2 onto the bacteria on the slide. Observing for the formation of bubbles. The positive reaction produced the bubbles.

Oxidase test- Used the sterile cooled loop to collect a small amount of bacteria from 18-24 h. colony and placed it on filter paper. Then used the dropper or Pasteur pipette to place a drop of *N, N, N', N'*-tetramethyl-p-phenylenediamine

dihydrochloride onto the bacteria on filter paper. Observing color changed within 10 seconds. The positive reaction was purple color.

Motility indole lysine – Used the sterile needle to inoculate the suspected bacteria once by stabbed in the semi-agar media through the bottom of the tube. After 18-24 h. of incubation at 35 °C, the motility and lysine decarboxylase and deaminase activity were read before testing for indole test. The positive result of motility was observed by the radiated movement from central of inoculation. If non-motile, the bacteria grew only along the line of inoculation. The presence of lysine decarboxylase caused the entire tube to revert to purple and if caused the yellow bottom with purple at the top of the tube indicated a negative test for lysine decarboxylase. The presence of lysine deaminase caused the top of the tube to turn deep red, the top remained purple in a negative test. The indole test was done by added 3-4 drops of Kovac's reagent to the medium. The positive reaction was red to pink and the negative reaction was yellow layer.

OF-glucose test – Used the sterile cooled to needle inoculate the suspected bacteria into the OF-glucose test medium tube by stabbed half way to the bottom of the tube. The medium tube was incubated at 35 °C for 48 h. The positive result for fermentation of glucose was turned to yellow color.

Simmons citrate – Used the sterile cooled loop to inoculate the suspected bacteria on Simmons citrate by streaked on the surface of the agar slant. The agar was incubated at 35 °C for 24 h. and observed the changed color of the agar. The positive result was deep blue color.

Triple sugar iron agar (TSI) – Used the sterile cooled needle to inoculate the suspected bacteria in the TSI agar by stabbed through the bottom center of the medium tube and then streaked on the surface of the agar slant. The agar was incubated at 35 °C for 24 h. and observed the changed color of the agar. If the organism fermented glucose but did not fermented lactose and/or sucrose, the slant became red and butt indicated the yellow color (K/A). If the organism fermented glucose, lactose and/or sucrose, the organism turned the phenol red indicator to yellow both in butt and in slant (A/A). Some organisms generated gases, which produced bubble in the medium. If the organism was non-fermenter, the slant indicated the red color while there was no change in the color of the butt. (K/NC).

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