CHAPTER I

GENERAL BACKGROUND

1. Introduction

Durian (*Durio zibethinus* Murr.), a tropical fruit native to Southeast Asia, is one of the most highly valued and desired fruits amoung Southeast Asians due to its distinct flavour and unique test. The common name 'king of tropical fruit' refers to two facets: the highly nutritional fresh and the big thorns on the skin (reminiscent of those in jackfruit), which apparently resembles the thorny thrones of the Asian kings of old. It belongs to the genous *Durio*, which is a member of the family Bombacaceae in the order Malvales and consists of 28 species (Brown, 1997; Subhadrabandhu and Ketsa, 2001). The family Bombacaceae is a strictly tropical family of plants native to both hemispheres. This species is also known by the synonyme, *D. accuminatissima* Merr**.** Durian fruit is a quite large weighs 2-4.5 kg. The rind which usually weighs more than half of the total fruit weight. The aril of durian is creamy yellow and emits an offensive odor. In spite of the unplesant smell, the soft, yellow-white pulp is cherished as a luxury food and aphrodisiac by South-East Asia. Its economic importance is increasing worldwide.

Thailand is major exporter of fresh and frozen durians. Beginning in May and extending through August, the durian seasonally announces its presence in Thai market and massive amounts of the rinds is disposed of as waste which could lead to environmental problems. It has been estimated that up to 4.14×10^5 tons of durian rinds is produced in nature each year (Center of agricultural information, 2004). However, it is often discarded as waste, which causes many environmental problems. Hences, the development in using of durian rinds are important issues.

A large amount of durian rinds can be disposed by several techniques. The most effective technique is to treat this waste by processing it as a new product, which depends on user's purposes. In the interest of the environment, attempts have recently been made to use this agricultural waste as a source of valuable material of

commercial importance; for example, polysaccharide gel (PG) isolated from fruit-rinds of durian have been found to be useful in preparation of food product as jelly and has also used as pharmaceutical excipients in, tablet, suspension and emulsion (Pongsamart *et al.*, 1989). Further investigations have also shown that water-soluble polysaccharide gel have antibacterial activities against certain strains of gram positive and negative bacteria (Lipipun, *et. al.*, 2002). Dressing film can be prepared from the water-soluble polysaccharide and *in vivo* study demonstrated that the dressing films enhance wound healing in pig and dog skin (Nakachat, *et. al.*, 2001 and Siripoksupkul, *et. al.*, 2004). Toxicity test of polysaccharide gel was determined, a high oral dose (2g/kg) did not induce severe toxicity in male mice and rats (Pongsamart, *et. al.*, 2001a). No toxic effect has been observed in subacute treatment in male mice (Pongsamart *et al.*, 1989 b.) and subchronic studies in male and female mice confirmed the consumptive safety of polysaccharide gel (Pongsamart *et al.*, 2001 b.).

Mastitis is defined as an inflammatory reaction of udder tissue to bacterial, chemical, thermal or mechanical injury, which causes major financial losses and milk wastage throughout the world. It's usually caused by bacterial infections, eventually damages the udder tissues (Yagi, *et. al.*, 2002). Until now, studies have focused on the economic aspects from which perspective mastitis can generally be considered as the most serious disease in dairy cow, however, cost are not the only negative consequence resulting from the infection. The environmental impact is also significant; milk is discarded, which means lower efficiency and hence greater environmental impact per produced liter of milk. Less milk is produced, which leads to an increased need for calf feed and meat production is also affected.

Searching new antibacterial agents from natural plants to replace and limit antibiotic uses is interesting. However, mastitis is not only an economic matter but also affects the environmental performance of milk products. Besides involving two types of milk losses (low-quality milk that has to be discarded because it is unfit for human consumption and milk that is never produced due to reduction in the productive yield), each mastitis case influences a number of flows at the diary farm that are involved in the environmental performance of milk production.

Organic dairy production is drawing increasing attention because of public concerns about food safety, animal walefare and the environmental impacts of intensive livestock systems. In organic dairy farming, a goal about improved animal welfare and avoidance of the use of chemicals has introduced restrictions in the use of antimicrobial for treatment of infectious diseases. In order to improve and minimize the use of antimicrobials and risk of antimicrobial resistance in organic farming.

World Health Organization has recommended all member countries to actively promote native medicines of their respective country (Kamb, *et. al.*, 2003). The use of conventional plant products described in ancient literature in modern medicine suffer from the fact that scientific evidence and explanation are lacking in the most cases. The present study, therefore, is an effort to investigate the antibacterial activity of polysaccharide gel (PG) from durian rinds against bovine mastitis. Accordingly, the antibacterial efficacy of PG as well as its antibacterial activity in bovine mastitis was studied.

The purpose of this study was to determine the inhibitory activity of polysaccharide gel against bacteria isolated from dairy cow mastitis. Since mastitis causing bacteria affect damaging not only dairy cows but also dairy industry. Extracellular susceptibility test by methods of agar diffusion susceptibility testing, broth microdilution testing and time kill analysis was investigated and varieties of several environmental factors with PG was used as a screening test on all bacteria isolates from cow mastitis. The factors examined in this study included pH, ionic strength, metal ion and temperature to broaden their application. PG prepared from different cultivars of durian fruit-hulls was also tested. The potential of polysaccharide gel serves as a natural antibacterial agent. In the present study, we aimed to investigate the antimicrobial activity of the polysaccharide gel using cow mastitis bacterial isolates of *Staphylococcus* spp., *Escherichia coli*, *Klebsiella* spp., *Pseudomonas* spp. and *Streptococcus* spp. as the test microorganisms.

2. Review of literature

1. Carbohydrates

Carbohydrates are one the basis of mass the most abundant class of biological molecules on Earth. The bulk of planet's carbohydrate is produced by photosynthesis, the process by which certain organisms, including plants, algae and some bacteria, assimilate atmospheric carbon dioxide and convert solar energy to chemical energy. The main sources of carbohydrates are plants. *D-glucose* is the major carbohydrate produced by photosynthesis. It is utilized by plants to built polymers such as cellulose and starch. Cellulose, the most abundant organic substance on earth is a structural component of plants (Sznaidman, *et. al.*, 1999). Carbohydrates are aldehyde or ketone compounds with multiple hydroxyl groups.

$$CO_2 + H_2O \xrightarrow{\text{Light}} (CH_2O) + O_2$$

Carbohydrates can easily be divided into three main groups called monosaccharides, disaccharides and polysaccharides. The general properties of these grouping are found below.

1.1 Monosaccharides Monosaccharides are the smallest units of carbohydrate structure. These are crystalline compounds, soluble in water, sweet to taste, and do not need digestion in order to be absorbed into the blood stream. D-glucose is the most abundant in nature. D-glucose is an aldose or aldosugar because it has a carbonyl group at the end of the carbon chain making it an aldehyde. If, however, the carbonyl is at any position other than the terminal position, then the sugars are ketoses or ketosugars.

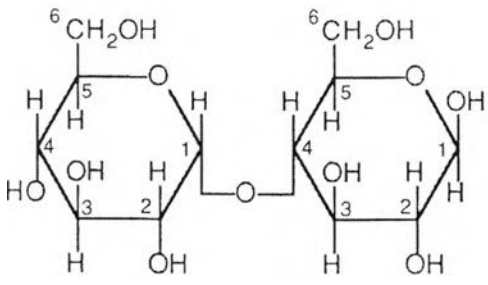
1.2 Oligosaccharides Oligosaccharides are polymer of 2 to about 20 monosaccharide residues connected by a glycosidic linkage. The most common oligosaccharides are disaccharides, which consist of two linked monosaccharide residues. Oligosaccharides are crystalline compounds, water-soluble, sweet to the taste, and must be digested to monosaccharides before they can be absorbed and used

for energy. Some of the more common disaccharides are maltose, lactose, sucrose, cellobiose and gentiobiose.

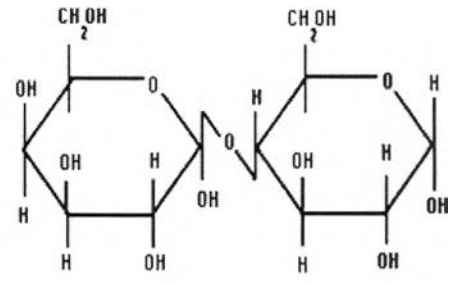
Maltose, which is a polymer of glucose residues. It is present in malt.

Lactose, a major carbohydrate in milk, is a disaccharide synthesized only in lactating mammary glands.

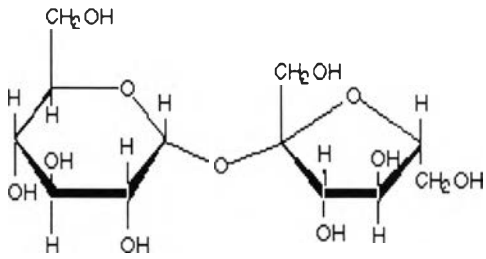
Sucrose, the most abundant disaccharide found in nature, is synthesized only in plants.



(a) Maltose



(b) Galactose



(c) Sucrose

Figure 1: Disaccharides (a) maltose (b) galactose (c) sucrose

1.3 Polysaccharides Polysaccharides are polymer that contain many (usually more than 20) monosaccharide residues. They are frequently derided into two broad classes: homoglycans or homopolysaccharides, which are polymers containing residues of only one type of monosaccharide, and heteroglycans or heteropolysaccharides, which are polymers containing residues of more than one type

of monosaccharide. They are found in the higher plants, in ferns and mosses, in seaweed, in fungi, in bacteria, and in animals, where they serve as a structural support and a food reserves. Many of the polysaccharides are hydrolyzed by specific glycosidases. Thus, cellulose is hydrolyzed by cellulase, and the starches and glycogens by the amylases and diastases. They are not sweet and must be digested before being absorbed.

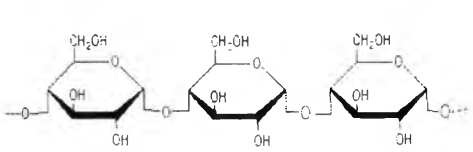
The main storage forms of polysaccharides are glycogen in animal cells and starch in plant cells. Both are deposited as granules in cells. Starch can be found in one of two forms: α-amylose or amylopectin.

Amylose consists of long unbranched chains of glucose attached to each other in α (1→4) linkages as shown in Figure 3. These chains may be from 3,000 kDa to 500,000 kDa molecular weight. α-Amylose readily forms hydrated micelles, and the chains form helical coils.

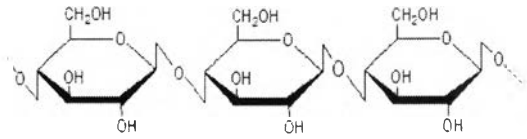
Amylopectin is highly branched with 24-30 residues/branch. The chains have α (1→4) linkages but the branch points consist of α (1→6) linkages as shown in Figure 6. Amylopectin forms colloidal or micellar suspensions, and its molecular weight can be as high as 100 million.

Glycogen is found to some extent in all animal cells but it is found in the largest quantities in skeletal muscle (1-2 % of the muscle wet) and in liver (up to 10 % of the liver wet weight under some conditions). On a fat-free basis, adipose tissue also has a relatively high glycogen content (about 1 %). Glycogen is structurally similar to amylopectin but is more highly branched with only 8-12 residues in a linear sequence between branches. Therefore, it is a more compact molecule than amylopectin.

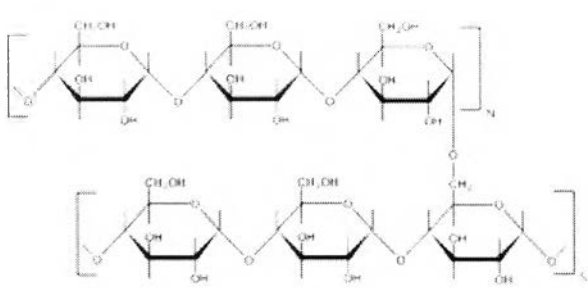
Pectin is a linear chain of 1,4-linked a D-galacturonic acid units, although almost all pectins contain a number of neutral sugars such as L-rhamnose, D-galactose and L-arabinose. Normally some of the galacturonic acid carboxyl groups are esterified with methyl alcohol.



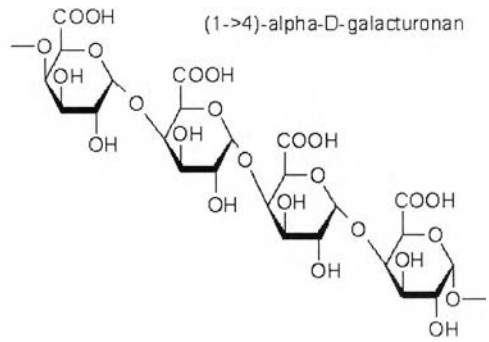
(a) Amylose: α-1,4 glucosidic bonds



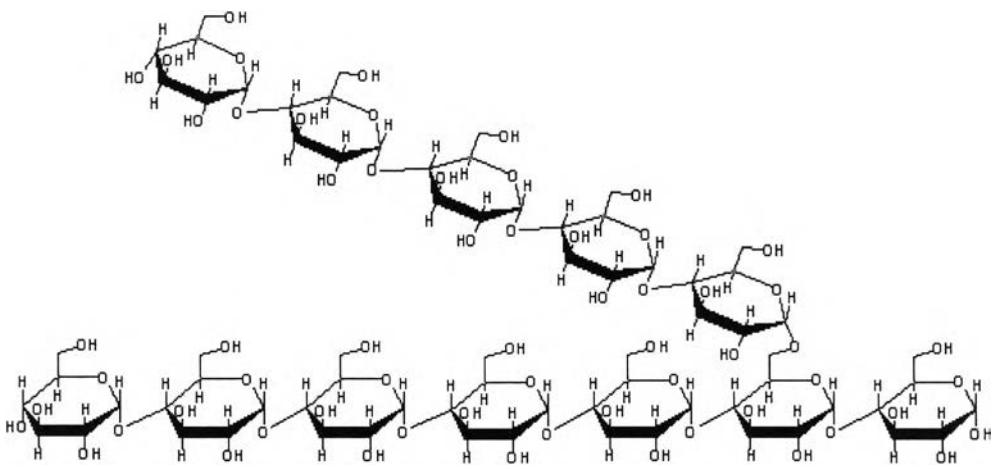
(b) Cellulose: β-1,4 glucosidic bonds



(c)Amylopectin



(d) Pectin



(e) Glycogen

Figure 2: Polysaccharides: (a) Amylose (b) Cellulose (c) Amylopectin (d) Pectin and (e) Glycogen

2. Durian

Durian (*Durio zibethinus* Murr.) is a member of Bombacaceae family. The family Bombacaceae is best known for showy flowers and woody or thin-shelled pods filled with small seeds and silky or cottonlike fiber. Durian is one member that differs radically in having large seeds surrounded by fleshy arils. They are large trees, able to grow up to 40 meters in height. The leaves are evergreen, opposite, elliptic to oblong and 10-18 cm long. Apart from variants of the word "durian" in native dialects, there are few other vernacular names, though the notorious odor has given rise to the unflattering terms, "*civet cat tree*", and "*civet fruit*" in India *"stinkvrucht "* in Dutch and "Thurian" in Thailand. Nevertheless the durian is the most important native fruit of Southeastern Asia and neighboring islands.

The family Bombacaceae also includes some other economically important plants, such as those belonging to the genera: *Ceiba, Bombax*, and *Salmalia*, which produce kapok (silk cotton); *Ochroma*, which is the source of balsa, the lightest wood; *Adansonia*, which produces edible fruit and wood fiber for paper making; and *Pachira*, which produces a valuable seed oil (Stanton, *et. al.*, 1996)

There are estimated to be 28 species in the genus *Durio* in Thailand. Only 5 species in addition to the durian bear edible fruits. These are *D. dulcis* Becc., *D. grandiflorus* Kost., *D. graveolens* Becc., *D. kutejensis* Becc. and ranked second to the durian in edibility; and *D. oxleyanus* Griff.

2.1 Edible Species

In addition to *D. zibethinus*, seven other species in the *Durio* genera are reported to be edible. Descriptions of these species, including *D. zibethinus,* are as follows.

Table 1: Descriptions of *D. zibethinus* species

Species	*Vernacular name*	*Distribution*
D. griffithii	*Thurian nok*	*Yala*
D. lowianus	*Chaarian, Thurian nok*	*Ranong, Trang*
D. malaccensis	*Due-yae- buu-kong, Thurian don*	*Yala, Narathiwat*
D. monsoni	*Thurian thuean, Thurian paa*	*Chumphon, Phangnga*
D. pinangianus	*Thurian*	*Yala, Narathiwat*
D. zibethinus	*Thurian*	*General*

D. zibethinus Murr: This is the common durian that bears fruit of very high quality and economically is the most important species. Because it is heterogenous, it exhibits a wide range of characteristic in tree form, fruit form and aril quality amongst a population of seedling trees.

D. lowianus: This species is found in the swamp forests of southern Thailand, Peninsular Malaysia and on the island of Sumatra. It is known in Thailand as 'Chaarian' (in *Ranong* province) and '*Thurian nok*' (in Trang province). The trees are occasionally cultivated because of their edible fruit. However, the round fruit only have a thin layer of fresh which is of inferior eating quality.

Mon thong and Chanee trees can be found in all center of durian cultivation in Thailand, ie in central Thailand (Nonthaburi province and its environs), estern Thailand (Rayong, Chantaburi, Trat, Prachin Buri province and its environs), and northern Thailand (Uttaradit province and its environs). The agro-climate in central and estern Thailand is dry, so Mon thong and Chanee cutivars and particularly dominant in those regions. Kan Yao and Kradum Thong cultivars are widely grown in Uttaradit, a province situated between Bangkok and Chiangmai. The agro-climate of Uttaradit is relatively more humid than that of central and eastern Thailand.

2.2 Medicinal Uses

The flesh is said to serve as a vermifuge. In Malaya, a decoction of the leaves and roots is prescribed as a febrifuge. The leaf juice is applied on the head of a fever patient. The leaves are employed in medicinal baths for people with jaundice. Decoctions of the leaves and fruits are applied to swellings and skin diseases. The ash of the burned rind is taken after childbirth. The seeds are believed to possess a toxic property that causes shortness of breath. The fruit is believed to have medicinal properties, restoring health to humans and domestic animals. Leaf, fruit and root extracts are used to reduce fever, and in treatment of jaundice, swelling and skin diseases.

2.3 Polysaccharide gel from *Durio zibethinus* Murr.

In the interest of environment, attemps have recently been made to use this agricultural waste as a source of valuable materials of commercial importance. A polysaccharide gel (PG) has been isolated from dried fruit-hulls of durian (*Durio zibethinus* Murr.). A process of PG isolation was performed based on the method previously described by Pongsamart and Panmuang (1998). Previous studies dedicated by Pongsamart and co-workers have isolated the water insoluble and water soluble polysaccharides from rinds, which have been found potential as pharmaceutical exipients. Their application such as a tablet binder, tablet disintegrator and gelling agent have been well reported (Pongsamart and Panmaung, 1998; Umprayn, Chanpaparp, *et. al.*, 1990a, b). Further investigations have also shown that the water soluble polysaccharides have antibacterial activities against certain strains gram positive and gram negative bacteria (Lipipun, *et. al.*, 2002). Dressing film can be prepared from the water soluble polysaccharides and in vivo study demonstrated that the dressing films enhanced wound healing in pig and dog skin (Nakchat, O., 2003 and Siripoksupkul R., 2004).

2.4 Sugar composition and properties of polysaccharide gel

Crude pharmaceutically useful polysaccharide gel (PG) extracted from the rinds of Durio zibethinus, are composed of pectin as the principal component and starch as a contaminant. PG is a water soluble polysaccharide, composes of sugars such as glucose (20.9%), rhamnose (4.8%), galactose (4.9%), xylose (0.4%), rhamnose (4.8%), arabinose (1.2%) and galacturonic acid (67.9%) (Hokputsa *et. al.*, 2004). The crude polysaccharides demonstrated a high viscosity enhancer.

3. Cow mastitis

Mastitis is defined as an inflammatory reaction of udder tissue to bacterial, chemical, thermal or mechanical injury (Philpot W.N., 1980). The word mastitis is derived from the Greek words *masos,* meaning "breast" and itis, meaning "inflammation of" inflammation of the mammary gland results from:

(1) trauma or injury to the udder

(2) chemical irritation or

(3) microorganisms

The degree of inflammation varies widely, from subclinical to various forms of clinical diesease, depending on how severely the udder reacts to the source of irritation.

In economic terms, mastitis can generally be considered the most serious disease of dairy cow. There are too many different bacteria involved and many of these are continually present. Antibiotic treatment has variety degrees of effectiveness and, for a variety of reasons, vaccination can only ever produce a partial reduction in incidence. Mastitis is commonly referred to under the four following categories:

1. clinical mastitis (CM): an udder infection which can be seen (different stages are peracute mastitis, acute mastitis and subacute mastitis), e.g. by clots in the milk, hardness, swelling, etc.

2. subclinical mastitis (sCM): an udder infection which show no external changes characterized by a change in milk composition but no sign of gross inflammation or milk abnormalities.

3. Chronic mastitis: exists for a long time and may continue from one lactation to another, but not severe.

3.1 Microorganisms causing mastitis

Over 140 different microorganisms can cause mastitis, and they live on the cow and in her environment. Thus, mastitis is the result of the interaction between cows, her environment and microorganisms (Figure 4). These microorganisms are microscopic forms of life and include bacteria, mycoplasmas, yeasts, algae, fungi and on rare occasions viruses. However, bacteria are the major cause of inflammatory infections in dairy cows.

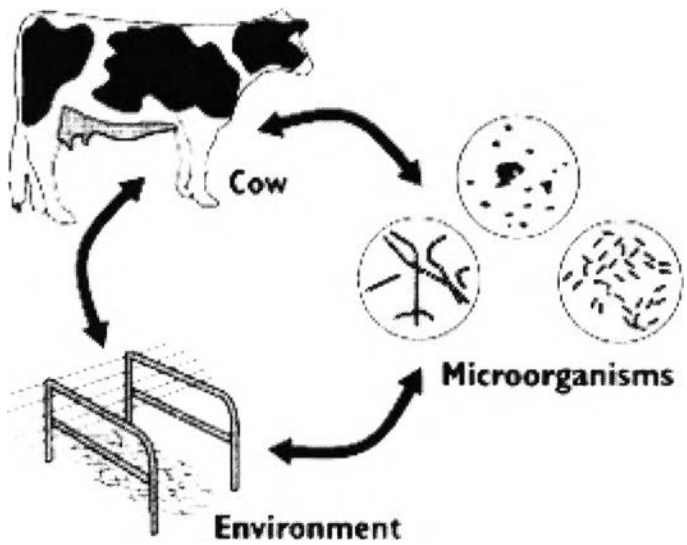


Figure 3: Mastitis results from interactions between the cow, her environment and microorganisms (Philpot W.N., 1980)

The microorganisms that most frequently cases mastitis can be grouped in four categories of pathogens as follows

(1) contagious

(2) environmental

(3) opportunistic

(4) others.

A pathogen is a microorganism that causes an inverse reaction in the animal it is infecting. Some pathogens infected very strong inflammatory reaction in the udder, which results in a very high somatic cell count (SCC). Other microorganisms are referred to as minor pathogens because they cause only a slight elevation in SCC.

3.1.1 Contagious microoganisms: Contagious pathogens are spread from infected to uninfected quaters and cows. The major sources of contagious microorganisms in milk from infected quarters. These microorganisms are spread from cow to cow during the milking process via milking machine clusters, milker's hands, and udder wash cloths. The most important contagious mastitis-causing microoganisms are the bacteria *Streptococcus agalactiae* and *Staphylococcus aueus* as well as the less pathogenic *Corynebacterium bovis*. *Mycoplasma bovis* are intermediate in size between bacteria and viruses, and are contagious pathogens but far less prevalent than *Staphylococcus aueus*, *Streptococcus agalactiae* and *Corynebacterium bovis*.

These bacteria establish subclinial infections of long duration, often called chronic infection, and the microorganisms are shed in milk in large numbers.

1. ***Streptococcus agalactiae***: a chain of spheres that reduces milk yield. The only reservoir of *Streptococcus agalactiae* is milk of infected mammary quaters. But they can be found on surfaces having recent contact with contaminated milk, including mlking equipment, milker's hands, and bedding mateials

These bacteria are shed in very high numbers in milk from infected quarters. It has been documented that one infected quarter of one cow in a 100 cow herd can elevate the bacteria count of bulk tank milk to more tham 100,000/ml. Spread of *Steptococcus agalactiae* to uninfected quarters occurs mainly during milking. In the absence of good udder hygiene and effective control measures, *Streptococcus agalactiae* can spread rapidly throughout a herd. Incomplete milking

of infected quarters may increase the severity of *Streptococcus agalactiae* mastitis, because large numbers of bacteria remain in infected quarters for spread to others cows.

Quarters infected with *Streptococcus agalactiae* typically have a high SCC that can markedly elevate the bulk tank cell count. Individual quarter SCC may range between 1 million and 10 million /ml. Clinical signs include slighly off – colored milk. This microorganism is sensitive to penicillin and can be eradicated from individual dairy herds. Subclinial infections can become chronic if not treated successfully with antibiotics resulting in non functional or blind quarters.

2. ***Staphylococcus aureus:*** These bacteria are not commonly found on healthy teat skin, but they readily colonize or grow in teat canel keratin. Teat skin chapping, resulting in lesions or sores, promotes colonization. *Staphylococcus aureus* growing in this sites are in an ideal location for infecting the udder, and are transmitted to uninfected quarter by milking machine teat cup liners, udder wash cloths, and milkers' hands.

Bacteria from infected quarter can be introduced to uninfected quarters to droplet impacted created by liner slios or other vacuum drops. Onces *Staphylococcus aureus* establishes an infection, a chronic inflammation results along with an elevated SCC. Firm, fibrotic aereas of scar tissue may be found upon (infections caused by *Staphylococcus aureus* are subclinical) in nature with periodic flare-ups of clinical symptoms. Such clinically infected quarters usually exhihibit moderate swelling and obvious clots upon forestripping. Chronic infections are extremely difficult to cure with antibiotic therapy because the development of scar tissue at multiple sites. These areas impede the distribution of antibiotics within the affected quarter after infusion and protect the staphylococci. As a result, antibiotic do not come in contact with the bacteria, the infection remains established, and the affected cow must be culled from the herd to prevent spread of the disease other animal.

3. ***Mycoplasma bovis*:** Mycoplasma are intermediate in size between bacteria and viruses and do not have a cell wall. Mastitis caused by mycoplasma

should be suspected when milk sample from cows with clinical symptoms, often multiple quarters, are negative after repeated culture using standard microbiologic methods. In addition, mycoplasma mastitis is characterized by: (1) a sudden onset; (2) formation of purulent secretion in affected quarters; (3) rapid transmission throughout the herd; (4) marked reduction in milk yield; and (5) resistance to antibiotic therapy.

4. ***Corynebacterium bovis*:** mammary gland infections with these bacteria are usually mild with a slight elevation in SCC, ranging from 200,000 to 400,000/ml. Mastitis out break caused by *Corynebacterium bovis* have been reported, most commonly in herd that do not practice postmilking teat dipping and dry cow therapy. Quarters infected with *Corynebacterium bovis* are less susceptible to infections by *Staphylococcus aureus* but are more susceptible to *Streptococcus agalactiae* and environment streptococci.

3.1.2 Environmental microorganisms

Environmental pathogens arise from the environment in which the cow lives. They enter the udder between milking when teats are exposed to mud, manure, and dirty bedding materials. The three primary groups of environmental mastitis-causing bacteria are

(1) Sreptococcus species, also know as environment streptococci (other than *Streptococcus agalactiae*) the *Streptococcus* species of greatest concern include *Stretococcus uberis* and *Streptococcus dysgalactiae*.
(2) Coliforms; the three coliforms include *Escherichia coli*, *Klebsiella pneumoniae*, and *Enterobacter aerogenes*.
(3) Enterococci; the most commonly isolate enterococci are *Enterococcus faecalis* and *Enterococcus faecium*

The prevalence of environmental mastitis is usually less than 5%, thus environmental mastitis often has very little effect on the bulk tank SCC. More over a high percentage of such infection become clinical and the milk is withheld from the

bulk tank. Environmental bacteria are abundant in the surrounding in which the cow lives, including manure, soil, bedding, feedstuffs, water and plant materials. Cows that are housed are at greater risk of infection with environmental than cows on pasture. The incident of clinical case may increase during winter months when animals are confined, but may also increase during summer months when exposure to the organisms is high. The reduction in prevalence of mastitis cause by contagious microorganisms, and the trend toward increased use of confinement housing. May be responsible for an increase in the proportion of new infections caused by the environmental microorganisms. Because bacteria are so widespread in the cows' surrounding, eradication is not possible on a practical basis.

1. Environmental Streptococci: within a herd, the percentage of quarters infected with environmental Streptococci is usual quite low, and most infection last less than 30 days. Approximately 18% of these infections become chronic and persist greater than 100 days, and 60 to 70% infection percent during lactation will result in clinical mastitis. About 40% of infections caused by environmental Streptococci present during lactation are cure spontaneously.

Streptococcus dysagalactiae is usually classified as an environmental streptococci, but it may also be have as a contagious pathogen. For example, this microorganism is readily controlled by teat dipping and dry cow therapy suggesting that transmission is sometime from cow to cow.

2. Coliforms: coliforms bacteria include the genera *Escherichia, Klebsiella*, and *Enterobacter.* These microorganisms live in mannure, bedding materials, soil, and poluted water. The most common causes of coliform mastitis are *Escherichia coli,* which are of animal origin, and *Klebsiella pneumoniae,* which are found naturally in the soil and the bedding material such as sadust, wood shavings, and straw. The number of coliforms in bedding material is usually lowest in the cold winter months and highest in the warm summer months.

Some coliform infections result from: (1) careless treatment procedure at drying off; (2) maintaining cows in a moist, dirty environment during the dry period; (3) calving in a contaminated area; and (4) not milking soon enough after

calving. Studies have shown that about 50% of coliform infection exist for less than 10 days and appear to cure spontaneously. Others studies have indicated that about 70% of infections persist less than 30 days, but some infection may persist more than 100 days, and can cause flare-ups of acute clinical mastitis.

3.1.3 Opportunistic microorganisms

Opportunistic microorganisms are the most prevalent microorganisms isolated from infected quaters but they cause only mild inflammation in udder tissues, and are referred to as minor pathogens. They live on the surface of the udder and teats in large numbers and are consequently a constant source of inflammatory infection. Other less common microorganisms also cause mastitis and include fungi, yeast, and algae.

This group of bacteria includes over 20 species of staphylococci other than *Staphylococcus aureus*. They may commonly referred to as *Staphylococcus* species or coagulase-negative staphylococci (CNS). These bacteria are of special interest because they are the most frequently isolated microorganisms in every herd; however, infections are usually mild. Clinical symptom are rare, and when they occur, such cases are mild, and local changes to the udder are limited to clots and flakes in milk.

The most common species of CNS are *Staphylococcus chromogenes, Staphylococcus hyics, Staphylococcus epidermidis, Staphylococcus simulans* and *Staphylococcus waarneri* are normal teat skin flora, while *Staphylococcus xylosus* and *Staphylococcus sciuri* appear to arise from the environment.

3.1.4 Other microorganisms

A wild variety of other microorganisms may also cause mastitis and include *Pseudomonas* species, *Arcanoacterium pyogenes*, *Norcardia* species, *Mycobacterium* species, and various bacilli, yeasts, molds, and algae. Infections with some of them are often due to poor treament procedures when infusing antibiotics.

Occurrence of infection is usually low, but outbreaks may occur when condition develop that increase exposure to them.

3.2 Isolation and identification of pathogens from milk

Microbiological examination of mastitic milk is done from aseptically taken quarter milk sample. A summary of examinations done in Thailand milk inspection laboratories in 2002-2003 is presented in Table2.

Table 2 Subclinical investigation in smallholder dairyfarms of Chiang Mai province during 2002-2003 (Boonyayatra S. and Chaisri W., 2004)

Herd	1	2	3	4
Average dairy milking cows (mean ±SD)	6.4±0.5	9.5±3.0	8.4±2.4	9.0±2.5
Incidence of subclinical mastitis (cases per cow-years at risk)	2.90	2.15	3.88	4.80
Prevalence of subclinical mastitis (%)	0-32.3	14.3-50.0	36.4-71.4	36.4-83.3
Intramammary infection pathogens (%)*				
Staphylococcus aureus	0	6.4	0	0
Coagulase-negative Staphylococci	17.7	27.0	18.8	13.1
Streptococcus agalactiae	0	0	0	0
Environmental Streptococci	0	32.4	11.3	49.5
Klebsiella spp.	0	0	1.25	0
Enterobacter spp.	0	2.7	5.0	3.0
Escherichia coli	0	0	0	0
Pseudomonas spp.	0	0	2.5	2.0
Corynebacterium bovis	5.9	0	6.3	5.1
Yeast	29.4	2.7	0	0
No growth	47.1	29.7	55.0	27.3

* Percentage of pathogens isolated from all subclinical mastitic milk samples from each farm

1. *Streptococcus* Genus. Gram-positive. Cocci, typically about 1μm in diameter, often in pairs or chains. Non sporing. Capsulation common. Facultatively anaerobic (the strictly anaerobic Gram-positive cocci are found in genera such as *Peptococcus, Peptostreptococcus* and *Sarcina*). Typically fermentative, sugars being metabolized usually without gas. Found e.g. as commensals and pathogens of man and other animals. Type species: *S. pyogenes.*

2. *Staphylococcus* Genus. Gram-positive. Cocci, about 1μm in diameter, often in clusters, some containing orange or yellow carotenoid pigments; non-motile. Facultatively anaerobic. Chemoorganoheterotrophic. Carbon sources include various sugars. Commonly halotolerant. Found e.g. as commensals and pathogens of man and other animals. Type species: *S. aureus*.

3. *Escherichia* Genus (family Enterobacteriaceae). The following refers to *E. coli*. Cells: single or impairs, typically motile (peritrichously flagellate) and fimbriate. Optimum 37°C. Respiratory under aerobic conditions; fermentation or e.g. nitrate respiration carried out anaerobically. Glucose is fermented (usually with gas) via the mix acid fermentation. Found e.g. a part of the normal microflora of the intestine in man and other animals; some strains can be pathogenic. Type species *E. coli*.

4. *Klebsiell*a Genus (family Enterobacteriaceae). Cells; single, pairs, short chains; capsulated. Non-motile. Found e.g. in soil, water and parasites/ pathogens in man and other animals. Type species *Klebsiella pneumoniae*.

5. *Pseudomonas* Genus. Gram-negative. Rods, 0.5-1x1.5-5 μm; most species have one/several unsheathed, typically polar flagella per cell, though *P. mallei* is non motile (i.e. it lacks flagella) and some species have sheathed flagella. Anerobic or facultatively anaerobic. Respiratory; many species can carry out nitrate respiration. Typically chemoorganoheterotrophic and nutritionally highly versatile; many strains will grow on organic salts with an organic carbon source, while some can grow chemolithoautotrophiccally. Found e.g. in soil and water, and as pathogens in man, other animals and plants. Type species: *P. aeruginosa*

3.3 Therapy and prognosis: Antimicrobial drugs

Antimicrobial therapy of coliform mastitis is problematic because the symptoms are caused in response to endotoxin. As the inflammatory response itself often eliminate the coliforms, the value of antibacterial therapy is questionable. Many reports have shown that there is no difference in cure rate whether one uses antibiotics

which are effective in vitro against gram-negative bacteria, or penicillin-G which should be infective. Coliform bacteria are eliminated at the same rate with or without "effective" antibacterial therapy actually comes at the stage when the endogenous factors are in the progress of eliminating the organism. At least 80% of mastitis case caused by *E. coli* recover spontaneously. *Kebsiella* mastitis has a consideraly poorer prognosis.

It is difficult to find an antimicrobial drug for gram- negative mastitis which has flavorable pharmacokinetics. Most *E. coli* strains isolated from mastitis are sensitive to trimethoprim-sulphonamide combination and enrofloxacin when tested in vitro on artificial media, such as Mueller-Hinton or ISB broth or corresponding agar plates; Klebsiella strains are susceptible *in vitro* to enrofloxacin. Using the intra vascular route, acceptable milk enrofloxacin concentration can be obtained. Enrofloxacin is also reabsorbed from the milk compartment. The metabolite ciprofloxacin accumulate gradually in milk. There is evidence that the antibacterial effect of fluoroquinolone may somehow be suppressed by milk. Coliforms are often susceptible *in vitro* to aminoglycosides, but these drugs cannot be recommended due to long withdrawal times, poor tissue penetration and unflavorable phamacokinetics in lactating cows.

As a whole, the use of antibacterials in coliform mastits is controversial. Antibacterials are often reccommended to be used in puerperal coliforms mastitis, as the cow's own defense mechanisms are weak at that time. On the other hand, experience from laboratory rodents and humans suggest that efective antibactial theapy might actually cause a sudden release of massive amounts of endotoxin. This can be detrimental to the host. There is no evidence on this therapy of bovine coliform mastitis.

3.4 Supportive therapy

The supportive therapy should be targeted at preventing these derangements. Survival largely depends on the degree of cardiovascular and metabolic derangement and on how well the therapy can correct these. Coliform mastitis is largely empirical. There are few published results from controlled therapy

trials. As the symptoms in peracute and acute coliform mastitis are triggered by endotoxin, elimination or neutralisation of the toxin is crucial. Frequent stripping combined with oxytocin-injections removes bacteria and horfully much of the endotoxin from the udder. As the disease culminates in fluid- and acid- base disturbance, diffuse coaglopathy, multiple organ disfunction, and shock.

1. The crude aqueous extract of *O. sanctum* (leaf) possesses some biologically active principles that are antibacterial and immunomodulatory in bovine sub-clinical mastitis. The aqueous extract of *O. sanctum* treatment reduced the total bacterial count and increased neutrophil and lymphocyte counts with enhanced phagocytic activity and phagocytic index (Mukherjee R. e*t al*, 2005).

2. An extract from the root of *Panax ginseng* was found to have stimulatory effects on neutrophils and lymphocytes from bovine peripheral blood and milk *in vitro* (Hu *et al.*, 1995; Concha *et al.*, 1996). More over, subcutaneous injection of a *Panax ginseng* activated the innate immunity of cows with subclinical intramammary infection of *S. aureus* (Hu e*t al.*, 2001a).

4. Composition and function of bacterial cell wall

4.1 Flagella

The surface filaments known as fagella are responsible for the motility of most bacteria. Some other bacteria use different structures for movement, including axial filaments. In addition to being observed under the microscope, motility can be demonstrated in various semisolid agar-containing media. Motile organisms are recognized by the visible spread of their growth pattern throughout the medium.

Motility can be significant in identifying a bacterial species. However care must be taken to distinguish true movement from the quivering to-and from motion know as Brownian movement. The latter is caused by a bombardment of the bacteria by molecules of the fluid in which they are suspended.

The present of flagella and their associated activity do not necessary correlate with other bacterial physiological properties. However, it appears that flagellation does bear a direct relationship to growth rate. Several factors may affect flagellation, among them the chemical composition of the medium, the pH, and the liquid or solid state of the medium. More flagellation occurs in liquid preparations.

Function: Bacteria cells benefit from flagella in the following ways:

(1) They can migrate toward environments favorable for growth and away from growth that might be harmful.

(2) They can increase the concentration of nutrients or decrease the concentration of poisonous materials near the bacterial surface by causing a change in the flow rate of environmental fluids.

(3) They can move flagellated organisms to uninhibited areas for colony formation. It has also been suggested that flagellated pathogens may more easily penetrate certain host defense barrier, such as mucous secretions.

4.2 Pili (Fimbriae)

Pili are surface filaments of varying diameters and lengths. In general, pili differ from bacterial flagella in several properties, including

(1) Their smaller diameter

(2) The absence of the wave like appearance so characteristic of flagella

(3) The apparent lack of association with an organism's true motility.

Chemical analysis of pili show them to be mainly protein. Specific homogenous protein subunits called pilin interlock and form the rigid, helical, tubelike pilus. The production of pili is under genetic control.

These filamentous surface structures have been found primarily in gram-negative bacteria. Included in this group are members of the genera *Branhamella, Escherichia, Klebsiella, Neisseria, Pseudomonas, Shigella and Vibrio.*

There have been reports of pili in numerous strains of the gram positive organism *Cornebacterium renale.*

Function: The functions of type I pili include

(1) attachment to most surfaces, cellular or otherwise

(2) formation of surface films of organisms (pellicles), which could enhance microbial growth in still-culture situations as important aids to establishing a disease process.

In this case certain pilliated bacteria attach to mammalian cell sufaces where they reproduce and produce toxins. Pili are also used as receptor sites by some bacteriophages (bacterial viruses) to inject their genetic material into a susceptible bacterial cell.

Another type of pilus, called the *F* or sex pilus, while similar inform to other pili, is different chemically. The sex pili are formed by doner bacteria containing plasmids for conjugative transfer of genetics material. Conjugative pili have been found among several bacterial species.

4.3 Cell walls

The need for some type of structure that would not only preserve a bacterium's shape but would also hold its cellular content together.

The rigid cell wall is the main structural component of most procaryotes. Its presence was first demonstrated by placing bacterial cells in very concentrated sucrose solution. The cellular membrane and its contents were seen to shrink away from an outer, enclosing rigid envelope as water from the inside of the cell diffused out into the sucrose under the influence of osmotic pressure. Thus, the bacterial cell's outer structural limit was defined.

Some of the functions of the cell wall are (1) to prevent rupture of bacteria caused by osmotic pressure differences between intracellular and

extracellular environments, (2) to provide a solid support for flagella, (3) to maintain the characteristic shape of the microorganisms, and (4) to regulate, to a certain degree, the passage of molecules into and out of the cell (molecular seiving). The sites of attachment of most bacterial viruses (bacteriophages) are on the cell wall. At distinct location, the wall is also modified to accommodate the surface appendages such as pili and spine.

The cell wall accounts for 20% to 40% of bacterium dry weight. Several factors affect this percentage, including the organism's stage of growth and its nutritional deficiencies. The strength of the cell wall is based on chemical and physical properties of complex molecular layer found only in bacteria, the peptidoglycan layer. However, not all bacteia cell wall contain peptidoglycan.

Various types of equipment and technique for isolating and characterizing bacterial cell walls have provided much information on their chemical composition and ultrastructure. The properties of bacterial cell walls is

1. Cell walls contain two simple sugars related to glucose, *N*-acetylglucosamine (NAG) and *N*-acetylmuramic acid (NAM). NAM occurs only in bacteria, including the cyanobacteria and the rickettsiae. NAG and NAM are connected by bridges of amino acids to form the mucopeptide or peptidoglycan layer of the wall. This backbone layer imparts mechanical strength to both gram- positive and gram-negative cell walls. It is the inner most path of the cell wall next to the cell membrane, and has been named the *murein sacculus*
2. Cell walls may contain a variety of natural or common amino acids that are used to build the necessary cross-linkages or bridges binding the peptidoglycan polymers (units) of the cell wall together. Included in this group are alanine, glycine, glutamic acid and lysine. Differences exist between the amino acid composition of gram-positive and gram-negative cell walls.
3. Bacterial cell walls also contain certain unquie amino acids, the atoms of which are arrange differently in space than those of most natural amino acids.

4. Certain organisms contain a molecule called *diaminpimelic acid.* It is found only in these bacteria in walls lacking lysine.

Certain structural and chemical differences exist between the walls of gram-positive and gram-negative bacteria. The walls of gram-positive bacteria are relatively thick (15 to 18 nm). Moreover, they are uniformly dense and exhibit a delicate odering of chemicals throughout their structure. In several gram positive species special polysaccharides are present in significant amounts, are bound to peptidoglycan layers, and contribute to cell wall structure. This substances include teichoic and teichuuronic acids, which also contribute to the wall's ability to bind heavy metals and control the autolytic (cell-digesting) actions of enzymes in certain cell walls.

The wall of gram negative cells are multilayered membranous structures and are chemically and structurally more complex than those of gram positive cells. They contain a wider range of amino acids and significant amounts of lipid, polysaccharide, and protein constituents. Combinations of these components from an outer layer or envelope that surrounds a thin peptidoglycan layer. The envelope is firmly attached to the murein layer and is connected to it by lipoproteins. Lipopolysaccharides (LPS) prevent the penetration of antibacteria substance, such as penicilin, that interfere with the formation of the peptidoglycan and are also known for their toxic (harmful) and antigenic (antibody forming) properties. The lipid portion of the LPS, refer to as *lipid A*, is an endotoxin that is known to cause the destruction of blood cells and fever during the course of certain infections. The polysaccharide portion of the LPS from envelope-associated antigens, which are of value in the immunological identification of some disease-causing gram-negative bacteria.

Several specialized functions and / or activities of the outer membrane of gram negative cells are reflected in its chemical composition. The structure contains a number of characteristic and unusual major proteins. Among these ae the *porins*, which act as pores by forming watery channels to allowed the passive passage of

various-sized molecules. Although most bacteria have rigid cell walls, one important group of organisms, mycoplasma, lack them. Because of the absence of a cell wall.

4.4 The plasma membrane

The plasma membrane lies just beneath the cell wall and separates it from the bacterium's cytoplasm. This structure, which is also called the *cytoplasmic membrane* or *cell membrane,* is an integral and indisensable functional part of all types of cells.

The eucaryotic cell, membranes partition the cellular space into compartments in which biochemical reactions occur. Structure similar, if not identical to the cytoplasmic membrane also surround intracellular eucaryotic organelles. The plasma membrane of the eucaryote is continuous or interconnected with the cell's internal membrane systems.

Chemically, the plasma membrane of bacteria consist of both proteins and lipids. Sterols, high-molcular weight lipids, are not found in the bacterial membranes with the exception of the mycoplasma, but they are common constituents of eucaryotic structures. The cell membrane contains several types of protein such as the cytochromes, iron-sulfur proteins, and the component of the electron transport chain. In addition, several types of enzymes are localized in the cell membrane and include the permease, mentioned earlier, and the biosynthetic enzymes that regulate the last steps in the formation of various cell wall macromolecules (peptidoglycans, teichoic acids, simple polysaccharides, and lipopolysaccharides) and membrane lpids. The lipids provide strength and other structural properties for the membrane. Membrane lipids are phospholipids. These molecule are amphipathic; they consist of region that are specially separated, with one end repelled by water (hydrophobic) and the opposite end attracted to it (hydrophilic). The hydrophilic end, which carries an ionic change and is polar, consists of glycerol attached to phosphate and other chemical groups. The non polar, hydrophobic end consists of hydrocarbon chains of fatty acids. With respect to the positive an arrangement of proteins on the phospholipids bilayer, it appears that proteins molecules are not arranged n an orderly

fashion, but actually penetrate the phospholipid region and may extend through it completely. Further, the protein molecules continually move and change their shape. The dynamic arrangement of the membrane molecules is refered to as the fluid mosaic model.

Membranes differ chemically in different cell types. Analysis have clearly shown differences in the types of lipids and in the protein: lipid ratios of membranes obtained from different kinds of cells. The enzyme compositions also vary considerably. In general, bacterial cell membranes comprise 10% to 20% of cell's dry weight.

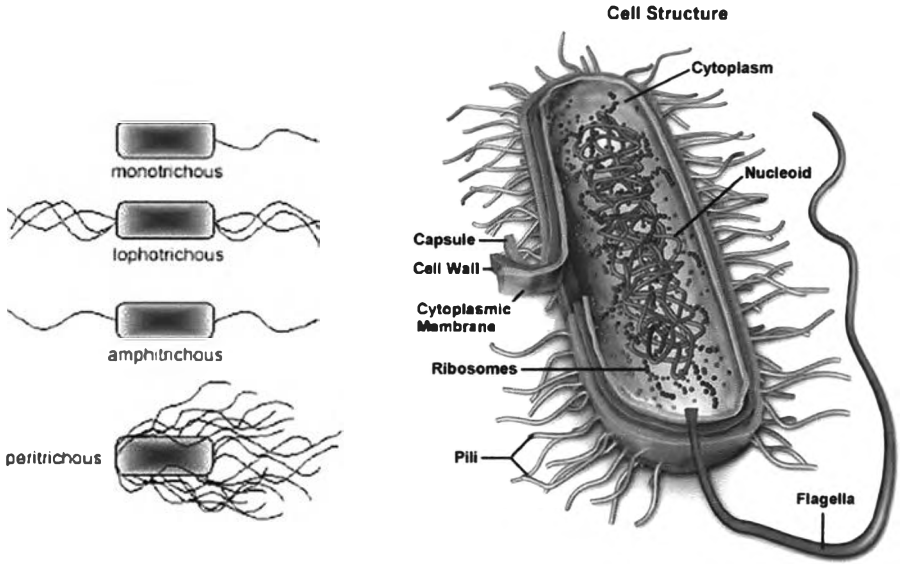


Figure 4: Type of flagella and bacterial cell structure (Brock, T.D, 1994)

5. Antimicrobial susceptibility tests

The primary purpose of antimicrobial susceptibility tests is to guide the clinician the choice of appropriate agents for therapy. The test is also provided accumulating data from which information for the suitable agents for empirical use can be derived. Antimicrobial susceptibility tests are used to evaluate an *in vitro* activity of new agents (Collin *et al.*, 1995).

In vitro antimicrobial susceptibility tests are depended on two roles, diffusion and dilution. Laboratory procedures involving diffusion susceptibility tests are common performed in agar media called agar diffusion technique.

5.1 Agar diffusion susceptibility tests

In general, Agar diffusion susceptibility tests are performed by inoculating a nutrient agar medium in a standardize manner and then applying the drug to be studied to the agar surface in some type of reservoir. The drug is allowed to diffuse into the surrounding medium. This expose the test organism to a continuous gradient of drug concentrations, with concentration diminishing as distance from the reservoir increase. After an appropriate period of incubation, there should be a zone of inhibited growth around the reservoir. The size of zone may be measured to determine the degree of susceptibility of test organisms (Lorian, 1991).

These tests depend on the ability of the antimicrobial agents to diffuse at predictable rates through the agar gel. The experiment work of Cooper (1964) provided a number of theoretical concepts that have led to a better understanding of variables that influence the formation of a zone inhibition in an agar medium.

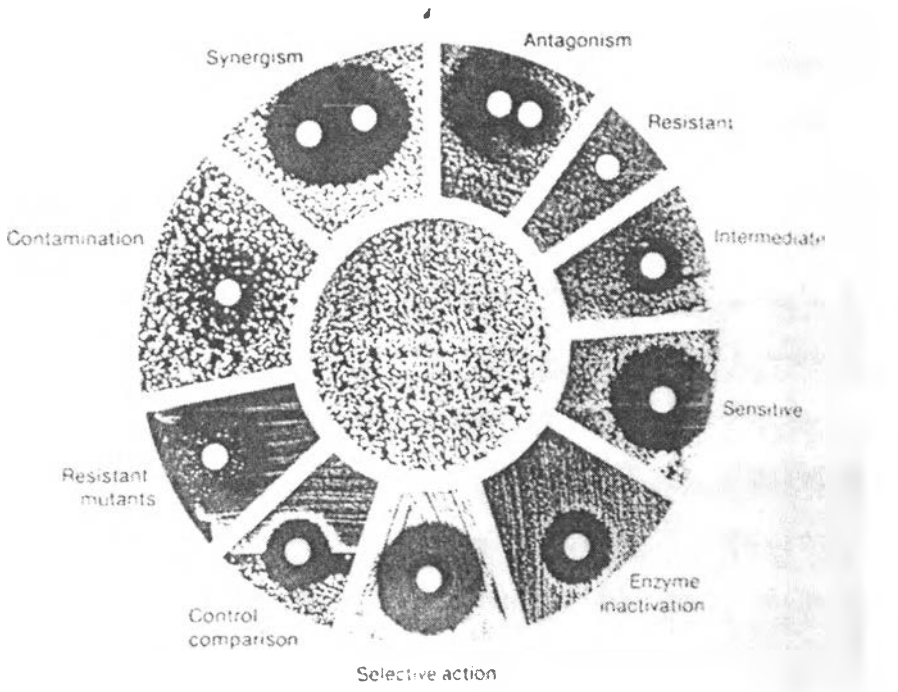


Figure 5: Antibiotic-sensitivity testing (disc diffusion test): types of zone which may develop around the disc (Lorian, 1991)

1. *Resistance* No zone: growth occurs right up to the disc.
2. *Intermediate* A narrow growth-free zone surrounds the disc.
3. *Sensitive* A wide growth-free zone surrounds the disc.

4. *Enzyme inactivation* A narrow growth-free zone surround the disc. Unlike the *intermediate* zone, the edge of the zone is sharply defined and it contains somewhat heavier growth with some normal-sized or relatively large colonies.
5. *Selective action* This result can be obtained e.g. when the inoculum consists of two different strains which differ in their degree of susceptibility to the given antibiotic. Close to the disc, the concentration of antibiotic is high enough, to inhibit both strains (narrow growth-free zone). Further from the disc (lower concentration of antibiotic) one strain is still inhibited while the other can grow.
6. *Control comparison* The half-zone obtained with a control strain (one side of the disc) is opposite the half-zone obtained with the test strain (other side of the disc). To make this comparison, the test and control strains are inoculated onto separate halves of the plate and the disc is placed between them.
7. *Resistant mutants* The inoculum contained a small proportion of mutant cell which were able to form colonies under conditions which inhibited non-mutants. The mutants are usually antibiotic-resistant cells. There is, however, another type of mutant which grows only in the presence of a given antibiotic and such mutants would also form colonies in an otherwise growth-free zone; for example, streptomycin dependent mutants contain non functional ribosomes which in the presence of streptomycin appear to be distorted in such a way that they become functional.
8. *Contamination* The inoculum contained a mixture of organisms, at least one of which is resistant to the antibiotic.
9. *Synergism* The two discs contain different antibiotics. The zone shows an inhibitory effect which is greater than the sum of the effects of each antibiotic acting alone; that is the antibiotics are acting synergistically.

5.2 Dilution method

Dilution antimicrobial susceptibility test methods are used to determine quantitatively in term of the minimum inhibitory concentration (MIC), the lowest concentration of antimicrobial agent required to inhibit the growth of an organism

isolate or that which kill it, the minimum bactericidal concentration (MBC) (Mahon *et al.*, 2000). The NCCLS 1997 document described the details of performing MIC and MBC tests by broth microdilution.

5.3 Broth microdilution susceptibility test

Recently, microdilution method commonly used in the susceptibility test as recommended in many studies (Nakajima Y., *et al.*, 2003, Chakraborty A. and Bratner A. H. 1999 and Giacometti A., Cirioni O. and Kamysz W. 2005). The broth microdilution test has been adapted to multi-well microdilution trays. Polystrylene trays containing between 80 and 100 wells are filled with small volumes (usually 0.1 ml) of two-fold dilution concentrations of antimicrobial agent in broth. Because of the large number of wells, several dilution as many as 12 to 15 antimicrobial agents can be contained on a single tray, that will be subsequently inoculated with one organism isolate. In the microdilution method mutiple plates may be conveniently produce by the use of apparatus for automatically diluting and dispensing solutions. The actual dilution factor used for preparation of the intermediate dilutions depends on the volume of inoculum derived to each well by the inoculating device. A growth control well is included on each tray. Growth may note as turbidity, a haze, or a pellet in the bottom of the well. After incubation, the tray is placed on one of several types of tray-reading devices (Mahon *et al.*, 2000). The end point of MIC, as note by Lorian (1991), MIC is the lowest concentration of drug at which the microorganism test does not demonstrate visible growth.

Several factors influence the outcome and reproducibility of broth susceptibility results. Most factors, media supplements, pH, incubation and inoculum size are likely affected as described in agar diffusion test.

5.4 Time-kill analysis

Time-kill analysis is methods which measures of the rate of the killing of microorganism by an antimcrobial agent as determine by examining the number of viable of organisms remaining at various intervals after exposure to the agent. It is an

extension of the MBC or MIC test. Test organism in mid-logarithmic growth phase is inoculated into several tubes of broth containing varying concentrations of antimicrobial agent and a growth control tube without drug. Most experiments are performed with a final inoculum of 10^5 to 10^7 CFU/ml. It is usually convenient first to adjust the overnight culture to match the 0.5 McFarland standard (NCCLS, 1997). These tubes are incubated. Then small aliquots are removed at specific time intervals (e.g. at 0, 4, 8, 12 and 24 hours) diluted to obtain countable number of colonies, and plate to agar for colony count determination. The number of organisms remaining in each sample is plotted over time to determine the rate of antimicrobial agent killing (Bartizal *et. al.*, 1997). Generally, a three or more $\log_{10}$ reduction in organism count and the antimicrobial suspension as compared with growth control indicates and adequate bactericidal or fungicidal response (Hoelloman *et al.*, 1998).

5.5 The assay of bactericidal activity

The MIC determination estimates the bacteriostatic or inhibitory activity of antimicrobial agents. The MIC, when performed according to the standards and references detailed, is reproducible parameter for a given antimicrobial agent against a variety of rapidly growing pathogens. In clinical practice, the MIC usually suffics for guilding chemotherapy. The success of *in vivo* antimicrobial action depends to a large extent on the host's defense mechanisms which ultimately sequester and kill the microorganisms that have been reduced by the bacteriostatic action of the chemoterapeutic agent. The main body of medical microbiology, clinical pharmacology, and infecious disease literature utilizers MIC data in studying the effect of antimicrobials and in establishing criteria for application in therapy.

For antimicrobial agents that possess the bactericidal action (mainly, aminoglycosides and β-lactams), it is sometime necessary to perform additional quantitative assessments of the killing effect on a given offending microorganism. The parameter termed the minimum bactericidal concentration (MBC) can be accomplished in several ways:

1. By estimating the MBC as a resultant of the MIC for an infecting organism;

2. By estimating the titer of serum of a patient receiving antimicrobial therapy that kills the infecting organism; i.e., the serum bactericidal titer or test (SBT)

3. By determining the number of surviving bacteria in a fixed concentration of the drug using the average obtainable blood level at the defined time intervals, i.e., the killing curve.

The assessment of bactericidal activity, although methodologically feasible, is fraught with microbiological-bacteriological phenomena and technical problems which require consummate understanding on the part of those who consider their application and individual who execute the assay.

5.6 Killing curve

The determination of the killing curve or killing rate is represented by a plot of a number of survivour in the host after administration of typical therapeutic regimen. It has ben used to evaluate and compare new drugs and study differences and changes in the antimicrobial susceptibilty of clinical important bacterial isolates. These determinations are used rarely for guilding chemotherapy and are mainly applied to experimental situlations in animal models, and apply more generally to classes of drugs. One concentration of antimicrobic is tested, usually that which is representative of an average obtainable level during therapy. At priodic intervals, usually at 0, 4, 12, and 24 hr of incubation, colony counts are performed and charted on semilog paper with the survivor colony count on the ordinate (logarithmic scale) and time on the abscissa in an arithmic scale. For example, when one compares the β-lactam antimicrobial agents with the aminoglycosides, the former are characterized by a slower dose-dependent initial bactericidal activity. The extent of the cidal action is related to the duration that serum level exceed the MIC. If levels fall below the MIC, there is immediate regrowth of microorganisms. Incontrast, amnoglycosides demonstrate rapid dose-dependent initial bactericidal activity, followed by a

bacteriostatic phase that can last several hours after serum concentration fall below the MIC.