CHAPTER 2

LITERATURE REVIEW

2.1 *Actinobacillus pleuropneumoniae*

Actinobacillus pleuropneumoniae is the major causative agent of pleuropneumonia in pigs. *A. pleuropneumoniae* is a member of Genus *Actinobacillus* under Family *Pasteurellaceae*, which comprised of 3 genera – 1) Genus *Pasteurella* 2) Genus *Actinobacillus* and 3) Genus *Haemophilus*, respectively (Reinier, 1999). *A. pleuropneumoniae* is a small gram negative, encapsulated rod with typical coccobacillary morphology. The organism does not grow on blood agar unless it is supplemented with Nicotinamide Adenine Dinucleotide (NAD) and shows satellitism around colonies of *Staphylococcus spp.* (Taylor, 1999). Staphylococcal streaking are normally required for primary isolation on this medium. This organism forms colony 0.5-1 mm after 24 hours incubation on blood agar in the presence of Staphylococcal colonies and is beta-hemolytic, particularly when the sheep red blood cells are used. Biovar 1 is the NAD dependent strains and biovar 2 is the NAD independent strains but requires the presence of specific pyridine nucleotides or the pyridine nucleotide precursors for its NAD biosynthesis (Bosse' et al., 2002, Reinier, 1999, Taylor, 1999). *A. pleuropneumoniae* produces an increased zone of hemolysis within the zone of partial lysis surrounding a beta-toxinogenic *Staphylococcus aureus* colonies (CAMP Phenomenon) and this phenomenon has been shown to be related to the possession of three cytolysins: ApxI, ApxII, and ApxIII.

A. pleuropneumoniae biovar 1 has been divided into 13 serotypes and recently, serotype 15 has been reported (Blackall et al., 2002, Frey, 1995). Serotyping has been extended into biovar 2 where six serotypes – serotype 2, 4, 7, 9, 13 and 14, respectively, have been identified (Frey, 1995, Schaller et al., 2001, Taylor, 1999). Serotype 2, 4, 7 and 9 of biovar 2 shared the antigenic characters with serotype 2, 4, 7 and 9 of biovar1, however, serotype 2, 4, 7 and 9 of biovar 2 lack of the *apxII* gene (Beck et al., 1994, Frey, 1995). The serological specificity is given by capsular polysaccharides and lipopolysaccharides (Frey, 1995, Tadjine and Mittal, 2001, Taylor, 1999, Ward et al., 1998). However, some serotypes show structural similarities or have identical LPS O-chains which explains the cross-reactions observed between serotypes 1, 9, and 11; serotypes 3, 6, and 8 and serotypes 4 and 7.

The capsular polysaccharides of most serotypes have been studied in considerable detailed and their composition is known (Belanger et al., 1995, Gottschalk et al., 1994, Inzana, 1995). Occasionally, the non-encapsulated forms of *A. pleuropneumoniae* might be found (Reinier et al., 1999, Taylor, 1999). The bacterial organism produces frimbriae 0.5-2 nm in diameter and 60-450 nm in length, which have been identified by the electron microscope. Outer membrane proteins of MW 43 kDa have been identified (Bunka et al., 1995, Gerlach et al., 1993, Gram et al., 1998, Osaki et al., 1997) and may vary with NAD availability. The iron-binding proteins have been identified and characterized and the genes encoding for its protein have also been sequenced (Gerlach et al., 1992, Tonpitak et al., 2000). The best-known extra-cellular products are the three cytotoxins belonging to the RTX family of toxins and named ApxI, ApxII, and ApxIII, respectively (Beck et al., 1994, Frey, 1995, Kuhnert et al., 1997). ApxI is a strong hemolysin of 105-110 kDa and is

presented in serotypes 1, 5a, 5b, 9, 10 and 11. It is encoded by the *apxICABD* operon consisting of *apxIC, apxIA, apxIB and apxID* which is the activator gene, the structural gene, and the two secretory genes, respectively (Beck et al., 1994, Frey, 1995, Jansen et al., 1993b). ApxII is a hemolysin of 103-105 kDa and is presented in all reference strains except in serotype 10. It is regulated by similar genes, however, secretion protein genes appear to be similar to those of ApxI (Beck et al., 1994, Frey, 1995, Leiner et al., 1999). ApxIII is a non-hemolytic cytotoxic protein of 120 kDa and is presented in serotypes 2, 3, 4, 6 and 8. It is regulated by the *apxIII* operon. The genes and many of the operon regulating ApxIII have been cloned and sequenced (Beck et al., 1994, Frey, 1995, Jansen et al., 1993a).

These exotoxins belonging to the family of pore-forming proteins named RTX-toxin (RTX representing repeats in the structural toxin) which play an important role in the virulence of a variety of human and animal gram negative bacterial pathogens including *A. pleuropneumoniae* (Kielstein et al., 2001, Kuhnert et al., 1997). The known operons are similar organized in a *CABD* pattern where *C* codes for the activation protein, *A* encodes the structural toxin, and *B* and *D* code for protein involved in the secretion of toxin. Not all RTX representative operon have a secretion of its own. ApxII, for example, takes advantage of the *B* and *D* protein encoded by the *apxI* operon (Frey, 1995). The common structural element of the RTX-toxins is a domain of glycine-rich nonapeptide repeats with consensus sequences L / I / F-X-G-G-X-G-N / D-D-X (Kuhnert et al., 1997). The total number of repeats bind calcium, which is necessary for cytolytic activities exerted by most of the RTX-toxins, and therefore is essential for toxin function.

The RTX toxin and RTX-like toxins can be detected in several species among pathogenic gram negative bacteria such as *Pateurella spp.*, *Actinobacillus rossii*, *Actinobacillus suis*, *Actinobacillus lignieresii*, and *Haemophilus parasuis* including *A. pleuropneumoniae* (Kuhnert et al., 1997). The RTX toxins, produced by *A. pleuropneumoniae*, consist of ApxI, ApxII, and ApxIII, respectively. These toxin are regulated by the *apxICABD*, *apxIICA*, and *apxIIICABD* (Figure 2.1), respectively (Frey, 1995). The gene profiles of *A. pleuropneumoniae* both of biovar 1 and biovar 2 strains are the same (Frey, 1995, Beck et al., 1994). A noteworthy of *A. lignieresii* is phylogenetically related to *A. pleuropneumoniae*, however, *A. lignieresii* lacks the –35 and –10 boxes in the promoter sequences (Schaller et al., 2000, 2001). *A. pleuropneumoniae* can be distinguished into four groups using toxins they produce. Group I, consist of serotype 1, 5a, 5b, 9 and 11, produces ApxI and ApxII toxins. Group II, consist of serotype 2, 3, 4, 6 and 8, produces ApxII and ApxIII toxins. Group III, consist of serotype 7 and 12, produces ApxII toxin. Group IV, consist of serotype 10, produces only ApxI toxin (Frey et al., 1995).

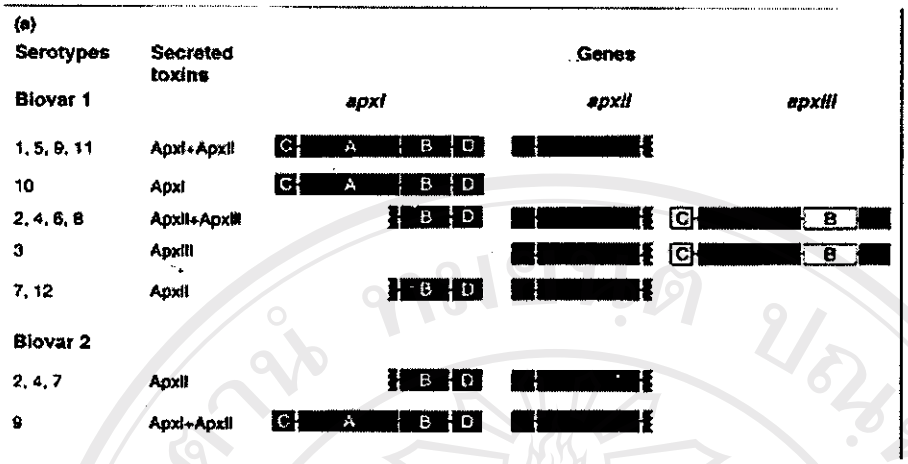

Figure 2.1 Map of the *apxI, apxII* and *apxIII* genes of *A. pleuropneumoniae* (Frey, 1995)

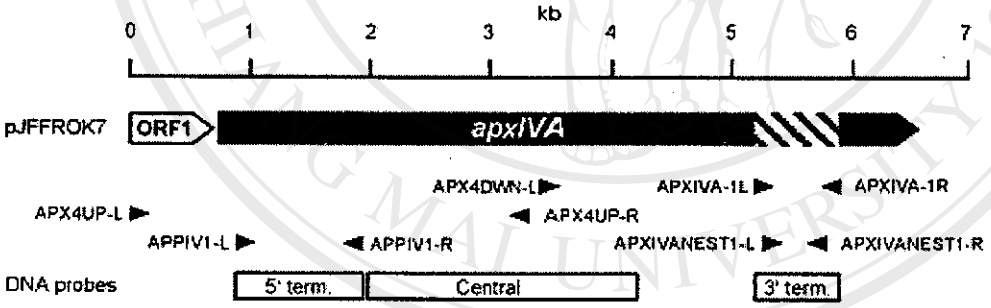

Figure 2.2 Map of the *apxIVA* gene of *A. pleuropneumoniae* (Schaller et al., 2001)

Recently, the *apxIVA* gene (Figure 2.2) encoding a new type of RTX determinant in *A. pleuropneumoniae* was described (Schaller et al., 1999), which can be detected in all *A. pleuropneumoniae* serotypes and seem to be species-specific.

The ApxIVA is expressed by *apxIVA* gene in pigs infected with all 15 serotypes of *A. pleuropneumoniae* (Blackall et al., 2002, Cho and Chae, 2001a, 2001b, Schaller et al., 2001). Schaller et al (2001) reported that the Southern blot hybridization and PCR amplification in the 14 serotypes reference strains of *A. pleuropneumoniae* (serotype 1 to serotype 14) could detect the *apxIVA* toxin gene. At the C terminal part of the *apxIVA* gene, variability in the DNA sequences encoding the nonapeptide glycine-rich repeats could be detected (Schaller et al., 1999). Only for *Actinobacillus lignieresii* show a weak reaction with the probe for the 5' terminal region of the *apxIVA* gene, however, other gram-negative bacteria are not (Schaller et al., 1999, 2001). With this respect, *A. lignieresii* is the most closely phylogenetically species related to *A. pleuropneumoniae*. The 3' terminal region of the *apxIVA* gene have been shown to be species-specific, which reacts to all 12 reference strains of *A. pleuropneumoniae* when using the 3' terminal region as a target for the probe. The primers designated from this region are also species-specific to *A. pleuropneumoniae* all of the reference strains and field strains but do not cross-reacted with the closely related bacteria including *A. lignieresii* when used as DNA template (Schaller et al., 2001).

2.2 Detection of *Actinobacillus pleuropneumoniae*

A. pleuropneumoniae is easy to demonstrate in the bronchial, nasal exudate, tonsils (Gram et al., 1996, 1998, Taylor, 1999) and pleuropneumonic lesion (Gram et al., 2000a) from freshly dead animals. Gram-stained smears of lung lesions contain numerous gram-negative coccobacilli (Reinier, 1999, Quin et al., 1999). *A. pleuropneumoniae* identities can be confirmed using the immunomagnetic separation (Angen et al., 2001b), immunohistochemical test (Takashima et al., 1999), detection

of serotype-specific antigens in lung extracts with a slide agglutination test (Hommez, et al., 1990, Sakpuaram, 1990), latex agglutination test (Inzana, 1995) and ELISA (Inzana and Fenwick, 2001, Klausen et al., 2001, Leiner et al., 1999, Nielsen et al., 1991). The nucleic acid from bacteria may be detected by a number of methods including the polymerase chain reaction (PCR) (Chiers et al., 2001, Cho and Chae, 2001b, Frey et al., 1995, Gram et al., 1996, 1998, Moral et al., 1999) and *in situ* hybridization (Cho and Chae, 2001a, Choi et al., 2001, Sirois et al., 1991, Ward et al., 1998). However, direct confirmation by PCR in tissue is not yet a routine method (Taylor, 1999).

Primary isolation of *A. pleuropneumoniae* from tissues and secretions may be carried out on 5% sheep blood agar with a cross-streaked of *Staphylococcus aureus* or *Staphylococcus epidermidis* as a nurse strains (Quin et al., 1999, Reinier, 1999). After aerobic incubation overnight, small colonies of *A. pleuropneumoniae* would appear in the neighborhood of the streak (NAD required) which is surrounded by a clear zone of complete hemolysis. This allows a rapid presumptive bacteriologic diagnosis. Altered blood agar ("chocolate agar") allows the growth of organism, however the colony characteristic is less distinctive on this media. The biochemical identification, were shown in Table 1, can be carried out by demonstrating the CAMP phenomenon, urease activity and the fermentation of manitol (Bosse' and MacInnes, 1997, Quin et al., 1999, Reinier, 1999). The selective medium have been described (Jacobsen and Nielsen, 1995) which has capability to culture organism from tonsils, an important site of carriage. However, culture may fail in very old chronic lesions, subclinical pigs or treated animals (Jacobsen and Nielsen, 1995, Taylor, 1999). In

peracute cases, it is possible to isolate the pathogens from other organs due to septicemia (Taylor, 1999).

Isolates may be confirmed as *A. pleuropneumoniae* by serological test using absorbed or monoclonal antibodies or by PCR (Bag et al., 1994, Enoe et al., 2001, Klausen et al., 2001). Serotype identification can be done using a PCR technique for the activator and the structural genes of the toxins (Beck et al., 1994, Frey et al., 1995) or more conventionally using monoclonal antibodies to particular serotypes (Inzana and Fenwick, 2001, Klausen et al., 2001, Leiner et al., 1999, Nielsen et al., 1991). The serotyping can be routinely achieved using rapid slide agglutination test from subculture on a medium enriched with serum or by the coagglutination test (Hommez et al., 1990, Sakpuaram, 1990). In many cases, the final identification can be achieved by agar gel diffusion test or by indirect hemagglutination test (Blackall et al., 1999, Sakpuaram, 1990). For serotyping organisms in tissue and isolated bacteria with latex particle test using capsular polysaccharide to sensitize the particles, is a critical evaluation of the earlier serotyping methods (Inzana, 1995, Taylor, 1999). Serotyping of the isolates is recommended for rapid confirmation of the bacteriologic diagnosis and is essential when vaccination policy is being considered (Frey, 1995, Taylor, 1999). The serotyping demonstrates the local distribution of serotypes and allows the epidemiological situation to be evaluated and the performance of specific serologic tests to be monitored.

Table 2.1 Differential characteristics of Genus *Actinobacillus* (Reinier, 1999).

Reaction	*A. pleuropneumoniae*	*A. lignieresii*	*A. suis*	*A. hominis*
V factor dependent	+	-	-	-
Alkaline phosphatase	+	+	+	+
Ornithine decarboxylase	+	-	-	-
Esculin hydrolysis	-	-	+	(V)
Urease	+	+	+	+
ONPG	(+)	-	+	+
Acid from Arabinose	-	V	+	-
Acid from Cellobiose	-	-	+	-
Acid from Galactose	V	V	V	+
Acid from Lactose	(+)	V	(+)	(V)
Acid from Maltose	V	+	+	+
Acid from Mannitol	+	+	-	+
Acid from Mannose	+	+	+	-
Acid from Melibiose	V	-	+	(+)
Acid from Raffinose	V	V	+	+
Acid from Salicin	-	-	+	V
Acid from Sorbitol	-	-	-	-
Acid from Sucrose	+	+	+	+
Acid from Trehalose	-	-	+	+
Acid from Xylose	+	(+)	+	+

[a] Symbols and Abbreviations: +, positive reaction; parentheses, delayed reaction; -, negative reaction; V, variable reaction; (+), most positive reaction; (V), most variable reaction

Detection of antibodies against *A. pleuropneumoniae* can be achieved in a number of ways. The complement fixation test (CFT) is suitable for this purpose (Enoe et al., 2001). In addition, simple serological methods for antibodies detection including enzyme-linked immunosorbent assay have been developed (Inzana and Fenwick, 2001, Klausen et al., 2001, Leiner et al., 1999, Nielsen et al., 1991). Antigens such as cell extracts or long chain lipopolysaccharide have been evaluated (Gottschalk et al., 1994) and testing with serotype-specific antigens allow the identification of antibody to a specific serotype (Taylor, 1999). However, detection of antibody to the toxins can indicate a measure of protection. Serum antibody determination can be used to determine antibody profiles of the herds or to demonstrate the colostral antibody (Taylor, 1999).

Agglutination tests are simple and quick methods for identifying and serotyping *A. pleuropneumoniae* isolates (Hommez et al., 1990, Sakpuaram, 1990). The rapid slide agglutination test is a popular method because it is easy to perform. Antiserum for agglutination test is collected from rabbit that has been inoculated with *A. pleuropneumoniae* antigen. The reaction is determined agglutination by visual inspection. Rapid slide agglutination test can usually be seen within a couple of minutes. A strong positive reaction in the form of clumping or agglutination occurred within a few seconds while stirring. In the negative reaction, no agglutination occurred (Sakpuaram, 1990). Although, the rapid slide agglutination test is easy to perform, nevertheless when compare with 2-mercaptoethanol-tube agglutination test and coagglutination test, rapid slide agglutination test was shown to be less sensitive and less specific than 2-mercaptoethanol-tube agglutination test and coagglutination test (Mittal et al., 1984). However, rapid slide agglutination test showed more

sensitive and specific than the indirect fluorescent antibody test (Rapp et al., 1985a). Consequently, rapid slide agglutination test is recommended in laboratory for a routine detection and serotyping of bacteria samples (Lo et al., 1998).

The genetic variability of a gene encoding for the outer membrane lipoprotein (*omlA*) genes (Osaki et al., 1997) are recently used for developing the PCR typing system (Gram et al., 2000a, 2000b). The *omlA* gene in each serotypes of *A. pleuropneumoniae* has been identified and sequenced (Bunka et al., 1995, Gerlach et al., 1993, Osaki et al., 1997). The sequence differences in the middle region of the *omlA* gene are used for dividing each serotype into five groups. The *omlA* I, consist of serotype 1, 9, 11 and 12. The *omlA* II, consist of serotype 2 and 8. The *omlA* III, consist of serotype 3, 6 and 7. The *omlA* IV, consist of only serotype 4. The *omlA* V, consist of serotype 5a, 5b and 10 (Osaki et al., 1997). However, serotype 4 can be discriminated from serotype 3, 6 and 7 by the size of PCR products. Serotype 8 is in had the *omlA* gene homologous to the *omlA* gene of serotype 2 (Gram et al., 2001b, Osaki et al., 1997). In some reports, the presence of the *omlA* gene encoding for serotype 8 resemble the *omlA* gene encoding for serotype 3, 4, 6 and 7 (Gerlach et al., 1993). Finally, investigation on the *omlA* gene encoding for serotype 8 have been confirmed to be resembled to serotype 2 (Gram et al., 2001b, Osaki et al., 1997). Earlier study, primers and probes are chosen from a part of the *omlA* gene common to all serotypes of *A. pleuropneumoniae* are analyzed by the 5' nuclease assay (Angen et al., 2001a). Problems of the PCR typing system by using the *omlA* gene was the variant strains of serotype 6 and 12 which have been detected (Gram et al., 2001a).

The PCR typing system have been developed for identification and detection of subclinical infected pigs (Frey et al., 1995, Gram et al., 1996, 1998). The

sensitivity and specificity of methods comparing with a routine bacterial cultivation method have been shown (Gram et al., 1996, Lo et al., 1998). The difference between the PCR typing system and other serotyping systems, such as latex agglutination test, is that PCR can show the serotype-specificity of each serotype but for latex agglutination test shows the serological-specificity. There were problems of cross-reaction between each serotype have been detected – such as IHA, which resulted in the false positive for sample identification (Blackall et al., 2002, Taylor, 1999).

The multiplex PCR for serotyping of *A. pleuropneumoniae* have been described (Gram et al., 2001a, Lo et al., 1998, Ward et al., 1998). The multiplex PCR assays have been used to analyze *A. pleuropneumoniae* using the highly conserved characteristics of the capsular polysaccharide export gene (*cpx*) and the serotype-specific characteristics of the capsular polysaccharide biosynthesis gene (*cps*) (Lo et al., 1998). The results are in agreement between each serotype and non-typable isolates with the exception of the rare serotype 4.

The PCR typing system using the *apx* and *omlA* gene, discriminating all of 12 serotypes of *A. pleuropneumoniae* with the exception between serotype 1, 9 and 11 and serotype 2 and 8. The serological cross-reaction between each of these serotypes are mentioned (Gram et al., 2001a). By using this PCR typing system, each serotype of *A. pleuropneumoniae* shows the *apx* / *omlA* gene pattern of their own.

2.3 Polymerase Chain Reaction (Erlich et al., 1992, Rolfs et al., 1992)

2.3.1 ***Principle of Polymerase Chain Reaction (PCR).*** Polymerase Chain Reaction (PCR) is an in *vitro* enzymatic amplification of specific DNA sequences or so called templates. The reaction is achieved by using oligonucleotide primers. Primer is typically short, single-stranded oligonucleotides which are complementary to the outer regions of known sequence. The oligonucleotides serve as primers for DNA polymerase and denatured strands of the large DNA fragment serves as DNA template. This results in the synthesis of new DNA strands, which are complementary to the parent DNA template strands. The new strands have defined 5' ends, which are the same 5' ends of the oligonucleotide primers, whereas the 3' ends are potentially ambiguous in length. The oligonucleotide directed synthesis of daughter DNA strands can be repeated if the new duplex is denatured and additional primers are allowed to anneal. A cycle of PCR consisted of 3 steps: template denaturation, primer annealing, and primer extension, respectively. The newly synthesized DNA strands serve as the templates for the next cycle until the last cycle. Half of the newly synthesized DNA strands from the second round of replication have 5' and 3' end that are defined by the annealing location of the oligonucleotide primers. The amplification of the DNA template strands occurs with the following patterns. There is always 1 copy of each of the original DNA template strands, PCR as outlined never amplify the full-length templates. The fragments of indeterminant length have one end defined by a PCR primer and the other end is of indeterminant length. There will be $(2^n - (n+1))$ copies of each fragment of defined length i.e. each end defined by the two PCR primers when n is a number of cycles. The desired PCR product will be a duplex of the defined length fragment. The original templates and

likewise the DNA template strands of the indeterminant length will not necessarily anneal with one another. Furthermore, as the number of cycles proceeds, the defined length fragments far outnumber of the others. Therefore, the original template, and fragments of the indeterminant length are most likely going to hybridize with defined length fragments. Consequently, the expected amplification of the desired defined length product with respect to the original template concentration "x" can thus be represented by the following formula:

$$[(2^n - (n+1)) - (n+1)]\,x \quad \text{or} \quad (2^n - 2(n+1))\,x$$

The interpretation of this formula is described as followed. First, for a given number of cycles "n" that makes "2^n x" total possible duplex. Second, for a given number of cycles there will be "2(n+1) (or 2n in our approximation) x" duplexes which are formed from either the original template, or a fragment of indeterminant length, along with a fragment of defined length (and represent an undesired product). Thus, the total concentration of desired product (duplexes with length defined by the PCR primers) will be "$(2^n - 2(n+1))$ x", where "x" is the concentration of the original duplex). Indeed, the practical amplification value is never achieved in practice. Several factors prevent this from occurring, including:

1. Competition of complementary daughter strands with primers for reannealing (i.e. two daughter strands reannealing results in no amplification).
2. Loss of enzyme activity due to thermal denaturation, particularly in the later cycles.

3. Even without thermal denaturation, the amount of enzyme becomes limiting due to molar target excess in later cycles (i.e. after 25-30 cycles too many primers need extending)
4. Possible second site primer annealing and non-productive priming.

2.3.2 *Thermal cycling parameters.* The birth of Polymerase Chain Reaction was invented by Saiki et al., 1985 and was patented by Perkin-Elmer Cetus. The thermal cycling parameters are critical to successful PCR experiment. It's comprised of 3 steps in each cycles of PCR including:

1. *Denaturing of template.* Denaturation is an initial heating to completely denature complex genomic DNA. Then, primers can anneal after cooling step. This step use high temperature to destroy hydrogen bond between leading and lagging strand of DNA. The denaturing step can be separated into 2 step: *initial denaturation* and *denaturation step.* Initial denaturation is always run before the denaturation step of the first cycle. It's accomplished at 95-100°C for 3-5 minutes. Denaturation step during the PCR assay (i.e. second cycle onward) is usually accomplished at temperatures of 92-95°C (usually empirically determined) for 20-30 seconds.

2. *Primer annealing temperature.* Annealing step use the optimized temperatures for primer that must be determined for each of the annealing site. This step reduces reaction temperatures to allow specific primer to randomly anneal to the complementary site. Additionally, the annealing temperature is characteristic for each oligonucleotide. It is a function of the length and base composition of the primer as well as the ionic strength of the reaction buffer. Practically, estimations of the

annealing temperature can be calculated from Tm of primers as described follow. These calculated-annealing temperatures are a starting point for the PCR experiment, but ideal annealing temperatures are determined empirically. In addition, if the temperature is too high, no annealing occur at all, but if the temperature is too low, non-specific annealing might increase dramatically.

3. *Primer extension temperature.* It's elongation step after primer annealing the complementary strands. The extension step is usually performed at 72° C, or the optimum temperature of the DNA polymerase. However, estimates of the elongation time can approximately calculate with the following formula: thirty seconds of the elongation time for 500 bp yield satisfactory results in this step. The length of time of the primer extension steps can be increased if the region of DNA to be amplified is long. Furthermore, at the last cycle, the final extension is performed to promote completely elongation. It's always performed at 72°C for 5-15 minutes after the last cycle.

These three components are performed in cycle of PCR. The number of PCR cycles is usually between 25-35 cycles. More cycles mean a greater yield of product. However, with increasing number of cycles the greater the probability of generating various artifacts (e.g. mispriming products) also increase. It is unusual to find procedures that have more than 40 cycles.

2.3.3 ***DNA polymerase for PCR.*** One of the important advances, which allowed development of PCR, was the availability of thermostable polymerases. This allowed initially added enzyme to survive temperature cycles approaching 100°C. The original method of PCR was performed with *Klenow*

fragment of *E. coli* DNA polymerase I. This enzyme denatures at lower temperatures than required to denature most template duplex. Hence, in each cycle of PCR, enzyme had to add to the reaction before beginning of new denaturing step, which is quite difficult. In Addition, the problem of this enzyme is reaction had to be moving from one waterbath to another to allow the individual steps of denaturation, annealing, and polymerization that in each step required different temperature. In order to solve the problem of changing PCR reaction to each different temperature waterbath, the machine that could shift their temperature up and down rapidly within an automated programming was invented. Subsequently, this machine is generally known as the thermal cycle machine or PCR machine. The thermostable DNA polymerases, which resisted denaturation at high temperatures, had to be used in the reaction (Table 2.2). The first thermostable DNA polymerase to be used was isolated from bacteria name *Thermus aquaticus*. This bacterium lives in a hot spring of Yellow Stone Park at temperature in excess of 85°C and it is the abbreviated name of *Taq* polymerase ("*T*" from *Thermus* and "*aq*" from *aquaticus*).

The *Taq* polymerase lack of the 3'-5' proofreading activity (Table 2.3). The fidelity of *Taq* polymerase depends upon the concentration of free Mg^{2+} and dNTPs, whether the four dNTPs are balanced, pH and heat damage to DNA template, among other thing. If a DNA polymerase lacks the proofreading activity, primers may be elongated despite a primer 3' terminus/template mismatching. In the case of *Taq* polymerase, the elongation will strongly depend on the type of mismatch. The following primer 3'-terminus/template mismatches significantly reduced PCR product yield after 30 cycles as indicated: C-C, G-A, A-G < 1 %; A-A to < 5 %. All other mismatches did not decrease product yield.

The synthetic rate of *Taq* polymerase depends on several factors: temperature, concentration of free Mg^{2+}, detergent, secondary structure of template, and concentration of dNTPs. Furthermore, the half-life of *Taq* polymerase is quite important, it depends on temperature such as at the temperature 92.5°C the half-life of this enzyme was 130 min. The increasing temperature to 95.0°C and 97.5°C, half-life of *Taq* polymerase was reduced to 40 min and 5-6 min, respectively.

The amount of DNA polymerase is one of the more important factors to be optimized for a particular assay. For most assays, the optimum amount of enzyme will be between 0.5 – 2.5 units in 50 µl of total reaction volume. Increasing of enzyme concentration occasionally lead to decrease specificity but sometime the excess enzyme concentration is essential in some sample.

Table 2.2 Thermostable DNA polymerases and their sources (Blaber, 1998).

DNA polymerase	**Natural or Recombinant**	**Source**
Taq	Natural	*Thermus aquaticus*
Amplitaq®	Recombinant	*Thermus aquaticus*
Amplitaq (Stoffel fragment)®	Recombinant	*Thermus aquaticus*
Hot *Tub*™	Natural	*Thermus flavis*
Pyrostase™	Natural	*Thermus flavis*
Vent™	Recombinant	*Thermococcus litoralis*
Deep Vent™	Recombinant	*Pyrococcus GB-D*
Tth	Recombinant	*Thermus thermophilus*
Pfu	Natural	*Pyrococcus furiosus*
UL Tma™	Recombinant	*Thermotoga maritima*

Table 2.3 Properties of DNA polymerases used in PCR (Blaber, 1998).

	Taq/ *Amplitaq*®	Stoffel fragment	Vent™	Deep Vent™	*Pfu*	*Tth*
95°C half life	40 min	80 min	400 min	1380 min	> 120 min	20 min
5' 3' exonuclease	+	-	-	-	-	+
3' 5' exonuclease	-	-	+	+	+	-
Extension rate (nucleotide/second)	75	> 50	> 80	?	60	> 33
Reverse transcritase activity	Weak	Weak	?	?	?	Yes
Resulting ends	3' A	3' A	> 95% blunt	> 95% blunt	Blunt	3' end
Strand displacement	-	-	+	+	-	-
Molecular weight (kDa)	94	61	?	?	92	94

2.3.4 *Buffer in PCR reaction.* Buffer is used to promote the activity of DNA polymerase in PCR reaction, the important components in buffer such as $MgCl_2$, Tris HCl, Tween, and etc. In addition to optimize reaction, it will be suitable to modify neither concentration of free Mg^{2+} nor concentration of dNTPs before all the other reaction components are optimized. If concentration of $MgCl_2$ modification is planned, it is advisable to store $MgCl_2$ stock solution at –20°C, due to the hygroscopic properties of $MgCl_2$, which make it impossible to weigh out the salt consistently. Numerous substances can promote (i.e. dimethyl sulfoxide, glycerol, formamide, polyethylene glycol and tween 20) or can inhibit (i.e. blood, phenol and proteinase K) the PCR process. Some components, particularly detergents – Triton x-100, are added to the reaction mixture with the enzyme, which is delivered in a specific storage buffer. This should be considered during optimization experiment when the enzyme concentration is modified.

2.3.5 *Magnesium chloride solution.* The Magnesium concentration may be varied from approximately 0.5 mM to 5.0 mM for optimum reaction. Mg^{2+} influences enzyme activities, increases melting temperature (Tm) of dsDNA, and forms soluble complexes with dNTPs, which is essential for dNTPs incorporation. The concentration of free Mg^{2+} depends on concentration of dNTPs, phosphate ions, and EDTA. Each of these compounds binds stoichiometrically with Mg^{2+}. Generally, low Mg^{2+} leads to low yield or no yield and high Mg^{2+} leads to accumulation of non-specific products (mispriming).

2.3.6 ***Deoxynucleotide triphosphates (dNTPs).*** dNTPs solution is comprised of dATP, dTTP, dCTP, and dGTP, respectively. Generally, a 10 mM stock solution of dNTPs is suitable for reaction and advisable to store at –20°C. Imbalanced dNTPs mixtures will reduce *Taq* fidelity. dNTPs stock solution should be neutralized to pH 7.0. The optimal dNTPs concentration depends on length of amplification product, $MgCl_2$ concentration, primer concentration and reaction stringency. On the other hand, dNTPs reduce free Mg^{2+}, thus interfering with polymerase activity and decreasing primer annealing. *Taq* DNA polymerase catalyzes dNTPs polymerization with higher fidelity at lower dNTPs concentration (10-100 μM) than those which are usually suitable for the optimal sensitivity of an analytical PCR (100-200 μM). In addition to a preparative PCR, where PCR products are reamplified to amplify ssDNA or dsDNA for sequencing or to synthesized labeled probes, it may be essential to decrease dNTPs concentration (20-40 μM), because of the high input number of the easily amplificable target.

2.3.7 ***Primers.*** Generally, primers used are 20 to 30 nucleotides in length. This provides for practical annealing temperatures (of the high temperature regimen where the thermostable polymerase is most active). Primers should avoid stretches of polybase sequence or repeating motifs – these can hybridize with inappropriate register on the DNA template. The G+C contents are ranging from 40 – 50 %. Inverted repeat sequences should be avoided to prevent hybridization to templates. Sequences complementary to other primers used in the PCR should be avoided so as to prevent hybridization between primers (particularly important for the 3' end of the primers). If possible the 3' end of the primers should be rich in G, C

bases to enhance annealing of the end, which will be extended. The distance between primers should be less than 10 kb in length. Typically, substantial reduction in yield is observed when the primers extend from each other beyond approximately 3kb.

The Tm of primer hybridization can be calculated using 3 commonly formulas as following:

1. This formula was determined originally from oligonucleotide hybridization assays, which were performed in 1 M NaCl, and appears to be accurate in lower salt conditions only for primers less than about 20 nucleotides in length.

$$Tm = [(\text{number of A+T residue}) \times 2°C] + [(\text{number of G+C residue}) \times 4°C]$$

2. The common wisdom is that the Tm is more like 3-5°C lower than the value calculated from this formula. This formula is useful for primers of 14-70 nucleotides in length.

$$Tm = 81.5 + 16.6 (\log 10 [J^+]) + 0.41 (\%G+C) - (600/l)$$

Where "$[J^+]$" is the molar concentration of monovalent cations (e.g. Na^+ from NaCl), and "l" is the length of oligonucleotide. (%G+C) is the actual percentage value and not a fractional representation (i.e. the value to insert for a primer which had 90% G+C content would be 90 but not 0.90).

3. This formula is useful for primers 20 – 35 nucleotides in length.

$$Tm = 22 + 1.46 ([\ 2 \times (G+C)] + (A+T)$$

The calculated annealing temperature is only a reference temperatures from which to initiate experiments. The actual annealing temperatures may be 3-12° C higher than the calculated Tm. The actual annealing temperature condition should be determined empirically. The highest annealing which gives the best PCR product should be used.

In view of these current knowledge it is recommended to keep these following rules in mind when designing oligonucleotides:

1) Rule of thumb: The reduction of the annealing temperature reduces the specificity of an assay but in some situations increases the chance to get any amplicon. Increasing of the annealing temperature increases the stringency of amplification so that in most cases, up to a temperature limit, a more specific product can be obtained.

2) The ratio of G/C to A/T should be approximately 1:1.

3) The oligonucleotides should not contain secondary structures. If secondary structures are present in the amplicon, the use of 7-deaza-2'-deoxyguanosine triphosphate (c7dGTP) can be helpful in certain cases.

4) The length should be between 18 and 26 bp for standard PCRs. In some cases, lightening of oligonucleotides contributes in some cases to a higher specificity. With allele-specific PCR assay, the shorter (14-16 nucleotides) primers are to be recommended.

5) The Tm values of the oligonucleotides should be between 65°C and 72°C. Conditions for a two step PCR (the annealing and elongation at the same temperature) improve with temperature approaching 72°C.

6) It makes sense to position the 3' end on triplets coding for conserved amino acids with nondegenerate codons.

7) When dealing with unclear template sequences, wobbling of the primer at critical points increases the probability of obtaining a product. Addition of an inosine base at the 3' end might further improve conditions.

8) 3' complementarity must be avoided for all primers under all circumstances. This is all the more true for multiplex PCR assays.

2.3.8 ***DNA Templates.*** Besides DNA, there may be other substances in the samples, which interfere in the PCR process. Many of these are mentioned above. Sometimes it is the purity of a sample, which limits the sensitivity of an assay. In some case, even the freezing of a sample just once or several times seem to affect reproducibility. For the clinical samples, it is important to be aware of the presence of the potential PCR inhibitory compounds such as EDTA, heparin, porphyrins, phosphate ions and related compounds.

EDTA and heparin are presented in blood samples as anticoagulants. They can be removed from blood samples by hemolyzed erythrocytes prior to lysis of the washed leukocytes. More than 0.25 mM $Mg_3 (PO_4)_2$ will precipitate in aqueous solutions at room temperatures. This means that small amount of PO_4^{2-} (e.g., from PBS buffers in alkaline preparations) will significantly reduce free Mg^{2+}, which might interfere the rate of reaction.

Samples, whether genomic DNA preparations or crude extracts, are never homogeneous solution. High-quality genomic DNA cannot even be handled with narrow pipette tips. Thus, it is sometimes useful to dilute an aliquot of the

sample before PCR analysis, and to use a larger, diluted sample volume in the reaction mixtures. One of the crucial factors of reproducibility is whether or not it is possible, to pipette a given sample identically at any date. The condition of storage samples such as freezing, heating and thrawing also influence this.

The optimum DNA content of a sample for PCR analysis depends on its purity and the underlying purpose of the assay. For genetic analysis, it is useful to analyze a sufficient standard amount of DNA (100-500 ng) of cell numbers to minimize the impact of nucleotide incorporation errors. For the detection of an unknown copy number of target DNA, as for example infectious agents, the maximum amount of sample DNA or cells which do not inhibit the PCR, has to be determined empirically.

2.3.9 ***DNA extraction from cells and tissues for PCR.*** Purity, homogeneity and DNA content of samples may vary considerably–largely depending on the native material itself and the method chosen to isolate or purify DNA. Many protocols have been described that yield DNA suitable for PCR. Criteria strongly influencing which protocol will ultimately be chosen are:

1) expenditure of time and manpower
2) diagnostic reliability
3) contamination risks
4) yield of amplifiable sample DNA

Samples containing less than approximately 70 ng DNA, the DNA purification through Phenol-Chloroform and the final ethanol precipitation step bear the risk of significant loss. On the other side, purified DNA will be the best storage

form for further analyses. Nonpurified samples after crude cell lysis cannot be quantified photometrically, but are useful to prevent loss of DNA.

Traditional protocols using proteinase K digestion and a number of often-lengthy subsequent steps after cell lysis were designed to isolate high quality, high molecular weight DNA for cloning procedures and for establishing DNA libraries. The demands that PCR places on the DNA template are different. As long as the DNA isolated is not extensively fragmented or nicked, so that the two primers employed in the PCR are not physically separated, the designed PCR should succeed. Proteinase K is still widely used in protocols describing the isolation of DNA for PCR. However, the time needed to obtain the desired template for PCR has been reduced considerably. The procedures, which take a day for DNA isolation, contain phenol extraction steps. The quick procedures omit these steps and also yield a DNA accessible to PCR. Whichever procedure is employed, care must be taken to eliminate substances known to inhibit PCR or at least to keep them at ineffective concentrations. Further, all measurements required avoiding cross-contamination or extraneous contamination must be realized.

Guanidinium salts are a chaotropic agent that destroy the three dimensional structure of proteins. Actually, Guanidinium salts are always used in RNA extraction, but there were applications for DNA extraction. It is used in the presence of a reducing agent to break disulfide bond and in the presence of a detergent such as Sarcosyl, is used to disrupt hydrophobic interaction. Guanidinium procedure generates high molecular weight DNA in application method with papain in the DNA extraction protocol of cartilage. Others application of Guanidinium procedures for DNA extraction have been explored.

2.4 Nested PCR. (Rolfs et al., 1992)

Nested primers are annealed internally in a per-existing amplification fragment. Extremely high sensitivity is to be expected when using a nested PCR. In addition, the nested primers serve as a specificity control for the external PCR fragment. For routine diagnostic procedures, however, the nested assays have the major drawback of introducing a high contamination risk. Quantitative artifacts from the external PCR will be enhanced during the nested PCR, thus impeding quantitative assays. These drawbacks may be partially circumvented by designing a "one-tube nested PCR" using two primer pairs of different melting points (Tm) in one reaction mixture. Their low Tm prevents annealing of the nested primers during the first cycles.

A difference in Tm of the external and nested fragment makes it possible to perform an exclusively internal PCR in the late cycles. The longer external fragment, which may have a higher G:C content than the nested fragment, will only be denatured at a slightly higher temperature than the nested fragment. This difference in denaturation temperature has to be determined accurately prior to performing analytical assay. In addition, the G:C content of the external fragment may be increased through the introduction of multiple G:C pairs("GC-clamp") at the 5'-end of the external primers. If a GC-clamp is used, the annealing temperature in the early cycles has to be low enough not to exceed the lower Tm of the external primers without the GC-clamp. After the initial cycles, GC-clamp sequences are introduced into the extension products, allowing for significantly higher annealing temperature. In summary, limiting outer primer concentration and using external fragments and primers with a higher Tm can perform "drop-in, drop-out nested

priming". In addition, for the synthesis of short PCR fragments (in one-tube nested PCR: the internal amplification product) lower denaturation temperatures give a higher yield of the intended product. Short PCR fragment can be amplified more effectively at denaturation temperatures between 87-90°C, while temperatures above 90°C reduce the yield. Large PCR fragment, on the other hand, require the usual denaturation temperatures between 92-95°C.

2.5 Multiplex PCR. (Rolfs et al., 1992)

The multiplex PCR is performed to amplify simultaneously several genomic regions from DNA templates of interest in one tube using a set of different primers. Simultaneous amplification of more than one DNA region of interest in one reaction mixture reduces time work, cost and the risk of contamination, since sample handling is minimal. In genetic analysis, the absence of one or more of the fragments might indicate a sequence deletion. The procedure appears to be less suitable for diagnostic purposes (e.g. detection of coliform bacteria and *E.coli*), particularly when used for the detection and differentiation of infectious agents in samples. However, application of multiplex PCR for the detection of infectious agent (e.g. viral agents) is mostly associated with a lower sensitivity of the assay. In contrast, the detection of mutations, deletion, rearrangements or insertion in single-copy genes is more suitable application of multiplex PCR. The different amplification products are not influenced in the same way by potential inhibiting factors of samples. This may lead to false-negative results for some amplification products, depending on which unknown factor is present in the samples. The experience with the multiplex PCR clearly emphasizes the importance of primer design and of the optimization of the amounts of primer

used. Even more than in a PCR assay using only one primer pair, care must be taken to avoid any 3' complementary between any of the primers. Hot start set-up strategy will probably significantly improve the specificity of multiplex PCR.

The following recommendation for the optimization or standardization of multiplex PCR assays can be formulated:

1. Thermocycler: only devices exhibiting constant physical properties should be used. Minute changes in the thermoprofile can lead to complete failure of the assay.

2. The length of the primers should be between 22 to 30 nucleotides – i.e. somewhat longer than in standard PCR assays. Primers with short sequences strictly must be avoided.

3. Multiplex PCR assays may require the addition of a cosolvent. Additions, such as dimethyl sulfoxide at the final concentration of 10% have proved to be advantageous in certain systems required high sensitivity.

4. Prolongation of the annealing time to approximately 1 min in the first five cycles, followed by a short annealing time (not longer than 30 seconds), at the highest possible temperatures, can increase the stringency of the assay.

5. Every additional new primer pair must be evaluated in the context of the whole assay.

6. Increasing the *Taq* polymerase amount up to 4-6 units/100 µl in the reaction mixture will in some systems improve sensitivity.