CHAPTER V
DISCUSSION AND CONCLUSION

In this study, azoospermia and oligospermia could be explained by a previous episode of orchitis in 22.3%, former bilateral cryptorchidism in 19.2%, abnormal karyotypes in 4.6% and Y chromosome microdeletions in 3.8%. The cause(s) of azoospermia and oligospermia in the other 50.1% was unknown. It has become more evident in recent years that a significant proportion (up to 35%) of such patients may have mutations and polymorphism of the androgen receptor (AR) gene. The increase in length of a trinucleotide repeat (CAG) tract in the transactivation domain of the AR has been reported to associate with an increased risk of defective spermatogenesis, especially in Asian populations [(44)]. As we did not study the androgen receptor, the conclusion as to whether defective AR played a significant role in our cases remains speculative. It is also remarkable that significantly more patients with azoospermia and oligospermia reported past or present use of cigarettes compared to males with normal semen analysis [(44)]. Further study should be done to ascertain whether cigarette smoking is gonadotoxic to male germ cells.

The frequency of abnormal karyotype in this study was within the previously reported range of 2.2-14.3% for infertile males [(17-19)]. The most common abnormality was Klinefelter's syndrome (66%, 4/6), which was in agreement with a previous study by Foresta *et al.* [(20)].

The levels of FSH, LH, prolactin and testosterone were undistinguishable in azoospermic/oligospermic males with or without microdeletions. This was in agreement with a large study by Tomasi *et al*[(132)], who found no difference in the function of pituitary-testicular axis in patients with and without Y-chromosome microdeletions. Levels of gonadotropins were significantly higher in azoospermic than oligospermic cases. This probably reflected a more severe impairment of spermatogenesis in the azoospermic cases.

There is a wide variation in the reported frequencies of Y-chromosome microdeletions; from 4.25% [(8)] to 23% [(100)] in azoospermic males and from 0.1% [(93)] to

8.5% [22] in oligospermic males, respectively. The reasons for this discrepancy might be due to many factors, such as the small sample sizes in most studies, the use of different inclusion criteria, the type and the number of primers used (5-118, mean 24.5 primers) and ethnic variations [33]. The prevalence of microdeletions increases with more strict patient selection and the higher percentage are found in patients affected by idiopathic severe oligospermia and idiopathic non-obstructive azoospermia [133]. As the present study excluded patients with obstructive azoospermia by history and clinical examination only, some of them could be included and, thus, lowered the prevalence of microdeletions in this studied population.

In this study, gene-based primers were used because STS primers were not specific for the genes that mapped the AZF intervals [134]. However, most previous studies used anonymous primers instead of gene-specific primers. The results of this study should, therefore, be more reliable and more informative than previous studies that used STS primers. The primers in this study amplified the only two known genes in the AZFa region, 4 representative genes out of 7 in the AZFb region and 4 of the 7 known genes in the AZFc region [135]. Primers selection was based on previous reports that showed deletions in Asian [136] and European population [35, 89]. It was possible that the present study underestimated the prevalence of Y chromosome microdeletions because not all known genes and other still unknown genes were screened.

The prevalences of Y chromosome microdeletions in Thai azoospermic and oligospermic males were 12.5% (5/40) and 1.25% (1/80) respectively. These frequencies were comparable to other recent studies in Japanese males [3/29 or 10.3% of azoospermic males in a study by Shimizu *et al* [137] and 12/157 or 7.6% of azoospermic and oligospermic males in another study by Kato *et al*. [138]]; Hong Kong azoospermic and severe oligospermic Chinese males (4/44 or 9.1%) [82]; Taiwanese azoospermic males (19/180 or 10.6%) [99], and New Zealand azoospermic and oligospermic males 7/127 or 5.5% [139]. This study confirmed earlier reports that azoospermic males had a higher frequency of microdeletions than oligospermic males, and that males with deletions in the AZFc region (50%, 3/6) were the leading group [6, 22, 95, 99, 121, 140]. Similar to a study by Foresta *et al*. [14], the second and third most common microdeletions were found in the AZFb (33%, 2/6) and the AZFbc (17%, 1/6) regions, respectively.

Four of the six patients had in common the presence of Y chromosome microdeletions confined to the AZFc regions, specifically in the DAZ locus. None had deletions in the AZFa region and only one had deletion involving a singe locus (*PRY* gene) in the AZFb region. This particular case had an average sperm count of 8.4 millions/ml but it was still doubtful whether his condition was due to *PRY* gene deletion or previous orchitis. Some studies, using STS markers, [134, 141, 142] reported that partial deletion in AZFb region might be associated with moderate oligospermia as seen in this case. However, there was no confirmation that deletion of *PRY* gene was solely responsible for abnormal spermatogenesis. A recent study by Stouffs *et al*.[143] suggested that the *PRY* gene probably played no role in spermatogenesis, but it might be involved in apoptosis of spermatids and spermatozoa.

Establishment of a multiplex PCR system often poses difficulties in making different primer pairs work together. In this study, some primers needed to be modified or redesigned to avoid primer competition and primer-dimer formation. Numerous optimization steps were also involved. HotStarTaq DNA polymerase was used to ensure a robust and more efficient multiplex PCR system. It is reassuring that all primer pairs successfully amplified normal male samples without amplification of the female samples. *SRY* gene was used as an internal control to ensure that there was no amplification failure, as this important gene was always present intact in all normal males. The strength of this study was that gene-based primers were used to amplify genomic DNA samples in a blind manner. Distilled water (blank) and female samples were routinely incorporated as negative controls and a normal male with no deletions was included as a positive control. Samples with microdeletions were checked at least 3 times to make sure that no PCR product was present. The drawback of this assay was that there was no sample with known location of Y chromosome microdeletions to serve as external control.

The Promega® kit, mainly used in the USA, is seldom used in Europe, probably because of its high cost (1,200 euros/ 23 reactions). The cost of this commercial kit is 5 times higher than the in-house multiplex PCR assays described in this study (3,780 Baht versus 750 Baht per sample). The commercial kit is also less versatile to implement, as it requires the use of specific equipment and reagents, such as Perkin Elmer thermocyclers and AmpliTag Gold DNA Polymerase [144].

In conclusion, an efficient, inexpensive and simple multiplex PCR protocol was developed to screen for Y chromosome microdeletions in infertile males by using somatic DNA from PBMC. The protocol gave reproducible results and was used successfully to detect Y chromosome microdeletions in 40 azoospermic and 80 oligospermic males.

Preliminary data on the prevalence of Y chromosome microdeletions and cytogenetic abnormalities in Thai infertile males in this study will be useful for counseling of patients and for future in-depth studies. Testing for Y microdeletions and chromosomal abnormalities is useful and should be done in all azoospermic males with no apparent abnormalities.