COMPARISON OF TWO DIFFERENT METHODS TO DETECT TELOMERASE ACTIVITY IN NORMAL AND NEOPLASTIC LYMPH NODES IN THE DOG

BY

FABIO VALENTINI

THESIS

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Adviser:

Professor Barbara Kitchell

ABSTRACT

One of the several differences between a normal and a neoplastic cell is that there is a physiological limit to the number of possible cell replications in the former, whereas it appears to be unlimited in the latter. For this reason, cancer cells are thought to undergo a phenomenon of immortalization.

The chromosome extremities contain structurally defined elements called telomeres. In normal somatic cells, each mitotic cycle leads to a reduction of telomere length until a critical point is reached. Telomere erosion to this critical length presents a signal for the cell to arrest further divisions and to undergo cellular senescence or to activate apoptosis. Telomeres that avoid critical shortening could, theoretically, replicate endlessly, thereby immortalizing the cell.

One mechanism to restore telomere length is the activation of a specific reverse transcriptase called telomerase. The telomerase is a ribonucleoprotein that contains a 9-base-pair RNA template to rapidly construct telomeric repeats. Upregulation of the telomerase enzyme provides immortalizing capacity to neoplastic cells.

Based on the hypothesis that telomerase is a specific marker of neoplastic tissues, the development of methods to detect its activity may represent an accurate, non-invasive diagnostic and prognostic tool.

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CHAPTER 1. INTRODUCTION

One of several differences between normal and neoplastic cells is that there is a physiological limit to the number of possible cell replications in the former, whereas it appears to be unlimited in the latter. For this reason, cancer cells are thought to undergo a phenomenon of immortalization. The chromosome extremities contain structurally defined elements called telomeres. Telomeres consist of tandem hexanucleotide repeats of the non-coding sequence TTAGGG. In normal somatic cells, each mitotic cycle leads to a reduction of telomere length until a critical point is reached. Telomere erosion to this critical length presents a signal for the cell to arrest further divisions and to undergo cellular senescence or to activate apoptosis. Telomeres that avoid critical shortening could, theoretically, replicate endlessly, thereby immortalizing the cell.

The most important mechanism to maintain telomere length is the activation of a specific reverse transcriptase called telomerase. The telomerase enzyme is a ribonucleoprotein complex that contains an RNA component within which lies an 11-base pair RNA template (5'-CUAACCCUAAC) complementary to the human telomere sequence (TTAGGG)n. Thus, new telomere sequences are quickly constructed. Up-regulation of the telomerase enzyme provides immortalizing capacity to neoplastic cells.

Based on the hypothesis that telomerase is a specific marker of neoplastic tissues, the development of methods to detect its activity may provide an accurate, non-invasive diagnostic and prognostic tool. The purpose of this project is to compare two different methods to detect telomerase activity in normal, hyperplastic and neoplastic lymphocytes in order to assess the best

method with regard to sensitivity and specificity. Our results should contribute to the development of a useful diagnostic tool for veterinary medicine.

CHAPTER 2. LITERATURE REVIEW

Telomere Nature and Function

The term "telomere" was coined by Muller and McClintock, etymologically derived from two Greek words: *telos* (end) and *meros* (part) (Blackburn, 1996). These two scientists were noteworthy because they were the first to understand the importance of telomeres in the maintenance of chromosome stability and integrity (Rhyu., 1995). As the telomere is the end of linear chromosomes, they are present only in eukaryotic cells. Telomeres give chromosomes stability and protect from enzymatic degradation and prevent fusion of chromosome ends.

The first molecular characterization of telomeres was completed in the 1970s (Blackburn and Gale, 1978) in the ciliate protozoon *Tetrahymena thermophilus*. Ciliates have been utilized extensively for telomere studies because they contain millions of independent DNA molecules and therefore, millions of telomeres in their macronucleus. It has been shown that *Tetrahymena* telomeres are made of TTGGGG sequences, repeated about 70 times. The G rich strand runs in the 5'-3'direction and extends for a brief tract beyond the C rich complementary strand.

As in *Tetrahymena*, the 5'-3' helix in vertebrates is G rich and extends 12-16 nucleotides beyond the complementary strand end (Blackburn., 1990). Telomeres give stability to chromosomes and protect from enzymatic degradation, prevent the fusion of the extremities with other chromosomes but, above all, their structure allow the double helix DNA, placed at the end of chromosomes, to be completely replicated.

Beyond their role in cell replication and chromosomal capping, telomeres have been proposed to participate in meiotic chromosome segregation and in the organization of the

nucleus. Observations of both somatic and meiotic cells suggest that the position of telomeres within the nucleus is highly specific and dependent on interactions of telomeres with the nuclear envelope (Dernburg *et al*., 1995). Telomeres sequences and length vary according to species (**Table 1**). In certain ciliates telomeres achieve 36 bp while in human beings and in other vertebrates the sequence TTAGGG is repeated to provide a total telomere length of 8-14 Kbp. In mice, telomeres can extend to150 Kbp (Dash *et al*., 1997). Within a given species, the estimated length of telomeric DNA varies from 2 to 20 Kbp, depending on factors such as tissue type and age of the organism (Jiao and Li, 2002). The number of repetitions varies between cells of the same organism, and even varies from chromosome to chromosome in the same cell.

Telomeres of a species may stabilize chromosomes of other species even if they have different sequences of bases. Telomere sequences of the yeast *Saccharomyces cerevisae* added, *in vivo*, to the ones of the ciliate *Tetrahymena thermophilus* resulted in appropriate telomere function.

To set up a functional telomere, a telomeric DNA is not sufficient but interaction with specific proteins is necessary. Among these, proteins that bind specifically to the single helix telomeric tract and proteins that bind to double helix telomeric repetitions have been detected; among the latter, the best characterized is Repressor Activator Protein 1 (RAP1) in *Saccharomyces cerevisae* and Telomeric Repeat Factors (TRF1 and TRF2) in human (Mondello., 1998).

In *Saccharomyces cerevisae* RAP1 is a protein that binds with a periodicity of about 18 base pairs along all the double strand telomeric DNA (in this species about 300 bp). Repressor activator protein one is thought to be a negative regulator of telomerase activity. Mutations in RAP1 that alter the DNA binding domain or alter RAP1 expression cause an uncontrolled

telomere elongation. Recent results indicate that the number of RAP1 molecules bound to telomeres is important for the regulation of telomere length. The cell can "count" the number of RAP1 molecules bound to DNA and based on that number can institute or repress telomeric repetition. It is presumed that RAP1 signaling is of a structural nature. Thus, binding of the appropriate number of RAP1 molecules to telomeres would make the chromosome extremities inaccessible to telomerase. There are other proteins acting in conjunction with RAP1 that contribute to uncontrolled telomeric elongation.

In human beings, two telomeric DNA associated proteins have been discovered. They are called telomeric repeat factors (TRF1 and TRF2). Telomeric repeat factor one is involved in the regulation of telomeric length and, as is the case in RAP1, acts as a negative modulator to prevent telomerase access to telomeres of the appropriate range of length. Previous work (Van Steensel and De Lange., 1997) showed that long term TRF1 overexpression in a telomerase positive cell line could lead to a gradual and progressive shortening of telomeres, on the other hand, telomeric elongation was induced by mean of the expression of a TRF1 dominant negative mutant that was able to inhibit the binding of a wild type TRF1 to telomeres (Van Steensel and De Lange., 1997). These experimental results identify TRF1 as an inhibitor of telomeric elongation and point out that this molecule is implicated in the negative feed-back mechanism that stabilizes the length of telomeres. The binding of TRF2 to telomeres seems to be necessary to protect the extremities of chromosomes from end-to-end fusion and to maintain the individuality of single chromosomes. If the bind of wild type TRF2 to telomeres is prevented by mean of overexpression of a mutant TRF2, the formation of dicentric chromosomes as a result of end to-end-fusions, is observed (Mondello, 1998).

The End-Replication Problem

The replication of the 3' end of the chromosome presents a problem in biology due to the polarity of DNA polymerase. One of the main reasons why linear chromosomes have telomeres is to circumvent the loss of DNA that takes place when the 5' terminus of a chromosome is replicated. This loss of 5' terminal sequence occurs because all known DNA polymerases require a pre-existing primer molecule in order to initiate DNA synthesis (Levy *et al*.,1992). In non-telomeric DNA replication, the strand that is continually replicated is called the "leading strand" whereas the complementary strand is called "lagging". To replicate the lagging strand, DNA polymerase III utilizes primers generated by RNA primases. This results in the creation of short DNA fragments called Okazaki fragments. The RNA primer template is subsequently degraded by DNA polymerase I, and the DNA fragments are extended and ligated by ligases to create a continuous DNA strand. The terminal RNA primer removal on the lagging strand, cannot be replicated and extended by this Okazaki fragment method. Thus, chromosomal DNA is shortened at the 3' extremity of all chromosome ends with each round of cell replication. Removal of this RNA primer results in the daughter strand being 8-16 nucleotides shorter than the parental strand. Therefore, there is a progressive loss of the end of chromosomes with each cell division. It has been proposed that this loss may play a role in the aging process (**Fig. 2-3**).

The enzyme telomerase solves this "end-replication problem" by adding DNA to the end of a chromosome by a process that is independent of the classical DNA replication machinery (Klapper *et al*.,2001). In addition, it now appears that the 3' single stranded telomere overhang is the template for telomerase activity. Telomerase is a specialized reverse transcriptase that contain an internal RNA template. The RNA template contains sequences that are complementary to the 3'overhang G-rich strand of the telomeric repeat. In normal somatic cells,

where telomerase is not active, the telomeres shorten with each cell division while in germ line cells, where telomerase is constitutively expressed, telomere length is maintained at a stable length. During addition of new telomeric DNA, the RNA template fragment aligns with the 3' overhang end of the chromosome, extending the end of the pre-existing DNA. To generate multiple repeats, the enzyme then translocates to the new chromosome terminus, realigns with the template region and the process is repeated (Klapper *et al*.,2001). As stated, a certain amount of telomeric DNA is lost during each mitotic division. Terminal restriction fragments analysis (TRF Assay) (Van Steensel and De Lange., 1997) demonstrated that chromosomes lose about 50 up to 200 nucleotides from the telomere per each cellular mitosis. This mechanism could represent a "mitotic clock" by which cells may "count" the number of division until critical telomeric shortening causes entrance into cellular senescence and blocks further divisions.

Telomerase Nature and Function

The standard mechanisms that permit chromosome duplication during cell division are unable to do a complete job. When DNA polymerases copy the two original or "parental" DNA strands, each new generated strand is shortened at one extremity. This is because of the polarity in DNA polymerase and the need for Okazaki priming RNA fragments, as previously discussed. When cells are not able to repair this defect caused by the duplicating mechanism, chromosomes would shorten continually (Greider and Blackburn., 1996). Experiments performed by these authors on telomeric DNA of *Tetrahymena thermophilus* detected an enzymatic activity that added sequences to the terminal DNA strands. Human telomerases work well on yeast telomere, suggesting that this functional conservation is not limited to lower eukaryotes (Blackburn., 1990). This RNA dependent enzyme is a terminal tranferase called telomerase. Telomerase is a ribonucleoprotein complex in which the RNA molecule contains a species specific sequence

complementary to the G-rich sequence of the telomere, which acts as a template for the synthesis of additional telomeric repeats.

Cellular extracts of *Tetrahymena thermophilus*, have high telomerase activity because of the large number of telomeres in the nucleus of this organism (up to 40,000 per macronucleus). When *T. thermophilus* is treated with RNAase A, the capability to extend the telomere ends is lost. This points out the importance of the telomerase RNA template fragment to telomerase complex function (Rhyu.,1995). When RNAase A treatment was performed on immortalized, telomerase positive HeLa cells, telomerase activity was also lost. Thus, the HeLa cell experiments also supported the observation of the ribonucleoprotein nature of telomerase (Rhyu., 1995). HeLa cells are a cervical carcinoma cell line developed in the 1940s. They are commonly used as a research tool in cancer and cell biology. HeLa cells are characterized by profoundly abnormal karyotype and morphological and biochemical differences compared to those of diploid cells (Holliday., 1995).

Telomerase is, therefore, classified as a reverse transcriptase, similar to the reverse transcriptase of Retroviruses. A reverse transcriptase is an unusual DNA polymerase that uses both RNA and DNA as a template; in viruses it is encoded by viral RNA and a molecule of enzyme is placed within each viral capsid during viral particle production. As the viral single strand RNA enters the cell, the reverse transcriptase contained in the capsid first makes a DNA copy of the RNA strand to create a hybrid DNA-RNA helix that is later used by the same enzyme to make a double stranded molecule of DNA. This double stranded DNA is subsequently inserted into the host genome (Alberts et al., 1995).

As stated previously, telomerase uses the 3' extremity of telomeric DNA as a starting point for extension by synthesis of repeated units of the template sequence contained in the RNA

subunit. This enzymatic complex creates multiple hexanucleotide repeats per cell replication cycle. Once the G-rich helix has been extended, the complementary strand is subsequently synthesized by standard DNA polymerase (**Fig.4**).

The absence of telomerase activity in normal somatic cells and its reactivation in neoplastic cells indicates an important role in cellular senescence and tumorigenesis. Telomerase is essential for chromosome end replication. It accomplishes this function with the aid of a variety of ancillary factors. Those ancillary factors guide telomerase in localization and in performance (Mondello., 1998). Telomerase reverse transcriptase protein and telomerase RNA do not seem to be the only two molecules involved in the ribonucleoproteic complex responsible for telomerase activity. Recently in *Tetrahymena termophilus*, genes codifing for two proteins (p80 and p95) associated to the telomerase RNA have been cloned.

The Catalytic Subunit of Telomerase

The most important and rate limiting component of human telomerase is the catalytic subunit hTERT and its expression correlates with telomerase activity (Liu *et al*.,2000; Nakayama *et al*.,1998). The catalytic activity that synthesizes telomeric DNA by copying the RNA template, is carried out by the protein subunit of the enzyme. The gene that encodes the catalytic subunit of telomerase (TRT) has been cloned (Lingner *et al*., 1997). This gene contains critical domains in which the introduction of a single amino acid substitution results in telomere shortening and cell death. This research points out that these domains are essential for the catalysis of telomeric elongation (Mondello, 1998). To date, hTERT mRNA has been detected in most of tumors with telomerase activities.

Restoring telomerase activity in human diploid cells is possible inserting the catalytic subunit (Bodner *et al*.,1998). To evaluate if telomerase activity was able to extend cell life span,

genetically modified hTRT proteins were inserted in normally hTRT (-) cells such as pigmented epithelial cells of retina and preputial fibroblasts. Based on the presence of telomerase activity in almost 90% of neoplastic cells, these authors compared telomerase expression in both the pigmented epithelial cells and preputial fibroblasts with control cells derived from lung cancer. Telomerase was highly expressed in both the experimental samples after the introduction of the genetically modified hTRT. Based on these results telomere length in normal hTRT (+) cells was measured to investigate the role of the catalytic subunit on its natural substrate, the telomere (Bodnar *et al*.,1998). In hTRT (-) cells, telomeres decreased in length passing from 1.3 up to 0.4 kbp whereas in the clones examined and modified by mean of catalytic subunit insertion, telomeres increased in length of 3.7 and 7.1 kbp respectively for retinal epithelial cells and for preputial fibroblasts. To evaluate the telomerase expression effect on normal cells life span, these authors compared the growth of hTRT (-) and hTRT (+) clones. Human telomerase reverse transcriptase (-) clones showed, as expected, a slowing in their growth leading to senescence. Human telomerase reverse transcriptase (+) clones showed a cycle of more than 20 duplications compared to the others (Bodnar *et al*., 1998). The telomerase complex also comprises hTERT-associated proteins such as Hsp90 and p23, which are required for activity of the enzyme and TLP-1, but the function of these proteins is still unknown (Jiao and Li Xin, 2002).

Neoplastic transformation of human immortalized cells was induced by mean of the ectopic expression of hTRT, after the insertion of an association of oncogenes such as SV40 large T and an oncogenic allele of the *ras* gene (Hahn *et al*.,1999). Primary rodent cells are easily converted in neoplastic cells by the coexpression of cooperant oncogenes. These experiments, however, often failed in human cells indicating some biologic difference between human and rodent cells as far as the expression of hTRT and neoplastic transformation. The few successes

obtained creating human neoplastic cells were due to the introduction of physical and chemical agents to reach immortalization or to the selection of rare spontaneously immortalized cells or to the use of whole viral genome (Hahn *et al*.,1999). An early entrance in the crisis phase was noted after just 10 cellular duplications in telomerase (-) human cell population expressing only large T and *ras*. No neoplastic masses developed when human cells expressing only large T or large T and *ras* or large T and hTRT, were introduced in immuno-deficient nude mice observed. On the other hand when the cells expressed all of the three genetic elements (large T, hTRT and *ras*), they quickly developed neoplasia if introduced in the same mice (Hahn *et al*.,1999). The conclusion of the work pointed out that the ectopic expression of a definite series of genes (large T, *ras*, hTRT) was sufficient to convert normal human cells. These results start to clarify the biochemical pattern that should be elicited to create a neoplastic transformation in normal human cells.

A potentially useful application in tumor diagnosis would be to develop specific monoclonal antibodies (MAbs) able to immuno-detect hTERT. Through the hybridoma technique clones secreting anti-hTERT antibodies were generated. The MAbs were hTERT-specific and reactive with native hTERT of human cancer cells presenting single band about 120 kDa, which was consistent with the weight of 127 kDa hTERT. In this work (Yang *et al*.,2001), the MAbs were used to detect hTERT in human cancer, precancerous and benign lesions and results showed that hTERT was expressed in about 80% of human cancers, consistent with those of telomerase activity and hTERT mRNA reported (Yuan *et al*.,2000).

Cellular Aging and Immortalization

Cellular Senescence

In the current literature cells that have finished their proliferative life span *in vitro* and have reached a terminal post-mitotic state are called senescent cells. This definition originated from the belief that the irreversible non-dividing state has a relationship with aging of the organism (Macieira-Coelho,1998).

Death after senescence must be differentiated from death by apoptosis or programmed cell death. Although senescence could come before the activation of a "suicide" program in the cell, a cause and effect relationship has yet to be established.

Programmed cell death (apoptosis) is well established in many multicellular organisms. Apoptosis "purifies" a tissue from cells that become useless or harmful for the organism. Similar phenomena occur also at subcellular level (suicide of mitochondria, i.e., mitoptosis). Mitochondria possess a mechanism of self-elimination mediated by the permeability transition pore (PTP). Massive mitoptosis results in apoptosis. At the supercellular level things are not so different. The massive apoptosis of cells composing an organ should eliminate the organ. This process is called organoptosis. An exemple of organoptosis is the disappearance of the tail of a tadpole when it becomes a frog. Obviously massive apoptosis in an organ of vital importance must lead to death of the entire organism and this is important if the organism is a member of a community of other individuals (Skulachev,1997). The programmed death of the organism is called "phenoptosis" (Skulachev,2002) and aging seems to be correlated with this phenomenon.

Many experimental works show the involvement of telomeres in the mechanism of cellular senescence (Levy *et al*.,1992). It has been suggested that the signal for cellular senescence originates from a single or a critical number of chromosomes whose telomeres have

eroded to insufficient length to function appropriately. These chromosome extremities would be recognized by the cell as double strand breaks and could trigger cell cycle control mechanisms that eventually block further cell multiplication as if damage to DNA was present. In favor of this hypothesis there is the observation that among the agents that transform human cells and determine the ability to overcome the senescence there is the SV40 T antigen. This molecule binds and inactivates p53, one of the most important factors involved in cell cycle regulation after DNA damage (Mondello., 1998). If in the cells was not present a mechanism able to fix this deficiency of the replicative system, chromosomes would shorten at each cellular generation and the genome would be eroded over time.

A correspondence between telomere length and replicative capacity of cultured fibroblasts has been well established. Cells from fetal tissues and from 90 year old donors have been assessed. Telomere length and lifespan of fibroblasts in culture, did not correspond with the age of the donor. However a relationship was found, instead, between the number of *in vitro* cellular divisions and the telomere length (Rhyu,1995). The knowledge of the role of telomeres and of telomerase in cellular senescence may be very helpful to understand the physiogenesis of aging and the pathogenesis of some diseases such as human progeria and Werner's syndrome.

Progeria is a rare genetic condition characterized by an appearance of accelerated aging in children. Its name is derived from the Greek and means "prematurely old." The disease is caused by an autosomal dominant inherited gene. Newborn progeria patients are apparently normal but after a variable time they begin to display evidence of rapid and progressive senescence. These signs of senescence include thin limbs, baldness, short height, and loss of subcutaneous tissue due to degenerative changes occurring at cutaneous, musculoskeletal and cardiovascular levels.

Werner's syndrome is caused, on the other hand, by an autosomal recessive mutation. This syndrome is much less serious than progeria. Affected people are normal for 20- 30 years with subsequent progressive changes such as juvenile cataract, diabetes mellitus, loss of subcutaneous adipose tissue of the limbs, osteoporosis and increased incidence of neoplasia (Hollyday,1995). In patients affected by these two rare diseases, the telomere length has been shown to be quite decreased compared to that of normal persons and a consequent chromosome instability and increased frequency of mutation has been noted (Dashe *et al*.,1997).

Like many important biological functions, aging also is mediated by several molecular mechanisms working simultaneously. These mechanisms include: telomere shortening due to suppression of telomerase at early stage of embryogenesis; age-related activation of mechanisms that induce the synthesis of heat shock proteins in response to appropriate stimuli; incomplete suppression of generation of reactive oxygen species (ROS) with inadequate scavenging of already existing ROS. Individually, none of these phenomena can kill the organism but may serve to weaken it, which can become crucial under certain conditions. Cells have several mechanisms to minimize the damage caused by super-oxide and other free radicals and ROS species. Among these defense mechanisms is the multilevel system of defense against ROS as well as mechanisms to prevent the accumulation of semiquinone (CoQH) and similar reductants. Additional enzymes decrease the intracellular oxygen concentration to reduce the rate of generation of superoxide. In addition to these cellular mechanisms, higher animals have physiological supracellular systemic effects, such as a decrease in lung ventilation and capillary constriction upon the transition from work to rest, when the demand for oxygen decreases (Skulachev,1997).

Heat shock proteins are a particular type of repair proteins whose contents increases considerably upon cell exposure to environmental conditions that denature protein molecules. In response to such stimuli, the cell activates the trimerization of heat-shock factor I (HSF I), a specific protein normally present in the cytosol in monomeric form. The HSF I trimer is transported to the nucleus where it activates promoters for heat-shock protein genes (Skulachev,1997).

Useful as a biomarker for senescence is also the β-galactosidase activity. Treating both hTRT (+) and hTRT (-) clones with this marker that has affinity for senescent cells, it has been seen that the majority of cells of the hTRT (-) clone expressed big amounts of β-galactosidase whereas scant amounts were expressed by cells of the hTRT (+) clone (Bodnar *et al.*,1998).

Cellular Immortalization

The majority of somatic cells of different mammalians have a limited life span in cell culture. After a defined number of divisions, cells incapable of further multiplications enter into a phase called "cellular or replicative senescence". In this phase cells are metabolically active and can remain in this state also for years if properly supported. However, cells in this phase do not respond to mitotic stimuli (Mondello,1998). The concept of immortalization is tightly linked to the concept of "senescence".

Telomerase plays a key role in cellular immortalization. Alternative mechanisms to stabilize chromosomes have been shown in experiments on the yeast *Kluyveromyces lactis* and on human immortalized cell lines in which telomerase activity was inhibited. However, for most cells re-induction of telomerase expression is required to allow escape from a senescent fate. These alternative mechanisms though, are less precise compared to the gradual telomeric repetitions elongation performed by telomerase (Rhyu,1995). Telomere length is prone to

shorten in all the somatic cells, whereas this does not happen in germ line cells that maintain an unchanged telomere length throughout many mitotic divisions (Norton *et al*.,1996).

It is possible to immortalize somatic cells by stabilizing their telomeres. Transfecting normal embryonal human kidney cells with the tumor antigen SV40T is possible to obtain an oncogenic viral protein that increases a cellular lifespan. These cells, eventually, reach a point of "crisis" in which most cells die. A small percentage of cells become immortal. During the period of somatic cell division *in vitro*, telomeres shortened progressively and no telomerase activity is detected. The few cells capable of overcoming the "crisis" phase to become immortal possess reactivated telomerase that restabilizes telomere length. This indicates that even somatic cells can multiply endlessly provided that telomere length is maintained, usually through the reactivation of telomerase enzyme (Dahse *et al*., 1997).

It has been hypothesized that *in vitro* transformed cells have only a limited lifespan extension and continue to lose telomere sequences (Counter *et al*.,1992). This loss may potentially play a role in the proliferative crisis seen of these cells. Telomerase activation and telomere stabilization usually occurs during the development of the immortal phenotype. To prove this hypothesis, the length and the amount of telomeric DNA, the incidence of dicentric chromosomes, and presence of telomerase activity during the lifespan of human kidney SV40 and Ad5 transformed or non-transformed embryonic cells has been measured. This research demonstrated that in the beginning, telomeric sequences were lost in similar amounts in all the cellular populations. The process of telomere loss continued uncontrolled in the Ad5 transformed populations. These cells had acquired only an extension of lifespan, and ultimately suffered death during crisis. In these non-immortalized populations, no telomerase activity was detected and the loss of telomeric DNA was associated with an increase in formation of dicentric

chromosomes. Populations of Ad5 transformed cells expressed telomerase and telomeric DNA stabilization was observed with minimal formation of dicentric chromosomes. Dicentric chromosomes increase in number over time in primary fibroblast cultures and this abnormality is common to both normal and transformed cells.

Chromosomes losing the protective effect of telomeric DNA are highly recombinant. Cells with shortened telomeres may experience an increased incidence of chromosome rearrangements that potentially could derive from end-to-end telomere fusion. In experiments using transformed populations these cells had an average of less than one dicentric chromosome per 50 metaphases but, during crisis, a significant increase in rearrangement frequency was noted. In this work three aspects of cellular immortalization were analyzed. First, extremely short chromosomes can induce an increase in dicentric chromosomes formation and cause entrance of transformed cells into a crisis phase. Secondarily, in immortalized cells telomere decrement ceases and the frequency of dicentric chromosomes stabilizes. Finally, it was noted that telomerase activity is not detectable in those populations that undergo telomeric loss but is present in immortal cells where telomere length is kept constant. Therefore it was supposed that telomerase expression is a requisite for cellular immortalization.

Of 35 immortalized cell lines, 20 showed expected telomerase activity. The other 15 did not demonstrate telomerase activity although they expressed long and stable telomeres. Hybrid cell lines, obtained by combining one telomerase positive and one telomerase negative cell line have been shown to undergo senescence even while expressing telomerase activity. This phenomenon is consistent with the hypothesis that telomerase activation is not essential for immortalization. Furthermore, some hybrid clones could achieve immortalization even in the absence of telomerase activity. Absence of telomerase activity in two SV40 immortalized cell

lines has been observed (Kim *et al*., 1994). These authors attributed immortalization to the long telomeres present in these cells. These results suggest the importance of telomere maintenance and stabilization in order for the cell to become immortal, and the importance of telomere shortening to potentially trigger cellular senescence.

Although telomerase activity was not detected in normal primary cell cultures or in viral oncogene transfected but non-immortalized cell lines, two hybrid clones of senescent cells expressed telomerase activity. This suggests that telomere stabilization is necessary but not sufficient to elicit senescence mechanisms, at least in the hybrid clones. Other organisms such as the yeast *Saccharomyces cerevisae* demonstrate telomerase-independent mechanisms to stabilize telomeres. *Saccharomyces cerevisae* uses recombination mechanism by which a non-telomeric portion of chromosome recombines in a different region of another chromosome. Research now focuses on investigating the presence of these mechanisms in human cells.

In mouse somatic cells, immortalization can occur spontaneously. The "mortal" phenotype of somatic cells is dominant to the "immortal" phenotype (Mondello,1998). In cell culture studies it has been shown that cells stop dividing due to the activity of antiproliferative mechanisms that contribute to a hypothetical model of cell immortalization. These antiproliferative mechanisms induce phases of cell culture kinetics called M1 (mortality stage 1) and M2 (mortality stage 2). Stimuli for the activation of M1 include DNA damage signals. The cell cycle regulatory genes p53 and pRb are involved in M1, (**Table 2**). Wild-type p53, along with other molecules with genome guardian functions, can activate proliferative arrest signals. These signals are activated by detection of telomeric repeat loss or structural variations due to telomere erosion (Hiyama *et al*.,1994). *In vitro* studies have also shown that M1 can be overcome after transformation with viral oncogenes such as SV40 (Morin, 1995). In M1,

telomeres are still functional even if cells are telomerease negative. Thus, telomeres continue shortening until the "mortality stage 2 or M2 phase is reached. Mortality stage 2 is activated when chromosomes lose the protective function of telomeres through cell replication induced erosion. Further cell replications are arrested to prevent subsequent chromosomal damage. In this phase, most cells are directed to the senescence pathway and then die (Morin,1995). Mortality stage 2 can be overcome by telomerase reactivation, chromosome extremity repair, and telomere length stabilization. This results in the emergence of immortal cell clones.

Genetic Changes Associated with Immortalization

Human somatic cells seem to have 3 terminal proliferative arrest (TPA) states that prevent immortalization. The first TPA state to be discovered was senescence. Senescent cells can remain viable for long periods of time, indicating that the senescence process is markedly different from any form of cell death. Senescent phenotype is dominant over the immortal phenotype (Bunn and Tarrant,1980; Pereira and Smith, 1983). This means that the limited lifespan of normal cells is a genetically programmed process and that immortal cells have lost the function of one or more putative senescence genes usually active in normal cells. The second TPA state is referred to as crisis, normal human cells rarely, if ever, become immortalized spontaneously *in vitro*. However, they can be immortalized reliably, although at very low frequency, following infection by DNA tumor viruses such as SV40 and papillomavirus (Linder and Marshall, 1990). SV40 has been the most commonly used agent to induce immortalization of non-hematopoietic human cells. This process consists of two stages, the first stage is a temporary lifespan extension. Following introduction of the SV40 DNA encoding the large T antigen and other oncoproteins, normal human cells become morphologically transformed and their proliferative lifespan increases by approximately 20-60 population doublings (PD) beyond the

point at which their normal counterparts enter senescence. Usually these cells enter crisis, during which no net population increase occurs. The second stage is the acquisition of an unlimited proliferative potential. Some of the cells in crisis may resume proliferation and become immortal.

Immortalization is a rare event, occurring at a frequency of between 10^{-5} (Shay *et al*.,1993) and 10^{-9}. Crisis therefore appears to be the final TPA barrier that needs to be breached before cells can become immortal. The abbreviation M1 and M2 have been suggested for senescence and crisis respectively (Wright *et al*.,1999).

Recent studies have shown the existence of at least one additional TPA state. Introduction of a dominant-negative mutant p53 gene permitted fibroblasts to proliferate for a limited number of population doublings beyond the point at which their normal counterparts became senescent (Fushimi *et al*.,1997). Evidence that this effect was due to loss of wt p53 function and not due to a p53 gain-of-function mutation was provided by experiments using a fibroblast cell line derived from a Li-Fraumeni patient. The IIICF fibroblasts contain one wt and one null mutant p53 allele. These cells escaped from senescence and proliferated for an additional 32- 35 PD when the wt allele was spontaneously deleted or mutated (Rogan *et al*.,1995). When the same heterozygous p53 wt/null-mutant IIICF cells were transformed with SV40 early region genes, the cells proliferated for an additional 60-70 PD beyond senescence, before entering crisis (Maclean *et al*.,1994). These studies indicate that fibroblasts in which normal p53 function has been abrogated escape temporarily from senescence before entering a TPA state. This “p53-minus TPA” state possesses the ability to support PD numbers intermediate between senescence and crisis.

Methods to Study Telomeres and Telomerase

By the time the important role of telomeres and telomerase in aging and cancer was understood, several methods had been developed to detect and study telomeric chromosomal regions and telomerase activity. Several protocols are available to detect TTAGGG telomere repeats *in situ*, including primed in situ (PRINS) (Therkelsen *et al*.,1995; Krejci and Kock,1998) labeling, or fluorescence in situ hybridization (FISH) with RNA- translated (Luderus *et al*.,1996) or nick- translated double stranded DNA (ds DNA) repeat probes (Henderson *et al*.,1996; Ijdo *et al*.,1991). Long oligonucleotides have proven effective telomere FISH probes (Meyne *et al*.,1994). FISH with short peptide nucleic acid (PNA) telomere probes yield detection efficiencies of nearly 100% (Lansdorp *et al*.,1996; Zijlmans *et al*.,1997; Martens *et al*.,1998).

Telomerase activity may be detected by several protocols. The first methods to be used were slow, cumbersome and based on incorporation of specific radioactive dNTPs into the telomeric repeats added onto the 3' end of a synthetic primer (Bilaud *et al*.,1997). These assays also were problematic because of very limited sensitivity. In 1994 (Kim *et al*.,1994) developed a polymerase chain reaction (PCR) based telomerase repeat amplification protocol, abbreviated as the TRAP assay. This methodology remains one of the most used protocols in telomerase research. The TRAP assay increased the speed of detection and drastically improved sensitivity by a factor of 10^4 over the prior radioisotope incorporation method. The TRAP protocol has been utilized to evaluate a large variety of human normal, reactive and neoplastic tissues. This early research supports the importance of telomerase activity detection as an early diagnostic tool as well as a prognostic indicator. The TRAP assay consists of two basic steps. A viable telomerase artificially extends polymeric repeats at the 3' end of a synthetic forward primer called TS. The telomeric products created in step one are subjected to PCR amplification through the addition of

a reverse primer. This results in amplification of a 6 base pair repeat ladder which is detected by gel electrophoresis. The TRAP assay, however, is fraught with technical difficulty and is prone to either false- negative or false- positive results. Telomerase- negative samples cannot be differentiated from telomerase- positive samples containing inhibitors of PCR amplification, thus giving false negative results. Furthermore, due to the repetitive structure of the PCR products, nonspecific amplification products resulting from staggered annealing of the reverse primer have been described (Kim and Wu,1997). The TRAP detection method relies on either radiolabelling with autoradiography or staining with ethidium bromide, silver nitrate or SYBR Green J and subsequent fluorescent imaging for quantification. Variations on the standard TRAP method include a fluorescent detection method in which a fluorescent TRAP reaction mix with a fluorescein- labeled TS primer are used.

Another approach to semiquantify the telomerase activity is the stretch PCR assay. As being more quantitative than the standard TRAP assay with this method it is possible to detect telomerase activity at the single cell level (Tatematsu *et al*.,1996).

A recently developed technique is the TRAP Enzyme-Linked Immunosorbent Assay (ELISA). This is a non- radioactive method that omits the gel separation step originally described for the TRAP method. An ELISA protocol is used to detect amplification products by a colorimetric method utilizing an ELISA-plate reader for semi-quantitation of results. The method seems to be useful for sensitive detection of telomerase activity and is especially valuable for studying large number of samples. Compared to the conventional TRAP assay, the TRAP ELISA is less time consuming because neither gel preparation nor electrophoretic separation steps are required. The sensitivity of the assay is equal or superior to that of the

conventional TRAP assay. The specificity observed is the same as seen with the conventional assay.

A very recent work (Xu *et al*.,2002) describes a novel method to measure telomerase activity by use of an enzymatic luminometric pyrophosphate (PPi) assay. This method is called ELIPA test. The ELIPA method is based on the elongation of telomeric repeats catalyzed by telomerase followed by the release of six PPi for each TTAGGG repeat. These PPi are later converted to ATP, which is detect by a luciferase bioluminescence system. The ELIPA was compared to the TRAP ELISA in a study of 42 lung carcinomas and 27 control normal tissues. The lower detection limits of ELIPA for telomerase activity was 5 cells whereas for TRAP ELISA was 10 cells; the sensitivity and specificity of ELIPA were 83% and 96%, respectively, whereas for TRAP ELISA were 71% and 96% respectively (Xu *et al*.,2002).

The advent of TRAP has allowed for the semi-quantitative detection of telomerase from limiting sample amounts. Due to the limited quantitative potential, TRAP is not very accurate in providing quantitative data. Real Time quantitative analysis is a recent method developed adapting the fluorescent-based TRAPeze XL telomerase detection kit with real time polymerase chain reaction. Quantitation of telomerase activity using real-time analysis is more precise than a gel-based approach because it relies on threshold cycle (Ct) values (Lynne *et al*.,2002). A threshold cycle value is the initial cycle in which PCR product is detectable. Ct values are determined during the exponential phase of PCR at relatively low concentrations of PCR product before saturation or plateau. This method ensure a more accurate quantification and high throughput capabilities improving reliability, assay time, and accuracy of quantitation (Lynne *et al*.,2002). In a different work the real-time quantitative TRAP (RTQ-TRAP) was compared with the conventional TRAP in 13 lymphoma samples (Hou et al.,2001). Telomerase activity

determined by the RTQ-TRAP method was 9-fold lower than that measured by the TRAP assay. This study suggested that the conventional TRAP assay may overestimate telomerase activity in tumor samples. The RTQ-TRAP assay may be thus a useful tool to rapidly and precisely quantify telomerase activity (Hou *et al*.,2001).

A recent study aimed to determine telomerase activity in colorectal carcinoma (CRC) and benign colon samples immunohistochemical stained for hTRT. The peroxidase method was used in the study. The conclusion of the study pointed out that increased telomerase activity in CRC, as demonstrated by hTRT immunostaining, is associated with poorer survival (Wei and Younes,2002).

In a different study (Yang *et al*.,2002) 3 hybridoma cell lines secreting anti-hTERT antibodies, designated as H4, G8 and A11 were generated. The affinity of G8 was stronger than that of H4 and A11. Sections from paraffin-embedded blocks of 127 cases of human cancer, 40 of pre-cancerous and 19 of benign tumors were in situ stained by G8 antibodies. The results showed that the neoplastic tissues were 80.31% positive and only 17.5% of pre-cancerous lesions presented weak positivity. Negative outcome came from benign tumors. The conclusion of the study confirmed the specificity of the G8 Mabs for hTRT (Yang *et al*.,2002).

Telomerase Activation in Neoplastic Cells

The loss of cell cycle control caused by mutations in the oncogenes and in the tumor suppressor genes, generally leads to cell death by apoptotic mechanisms but sometimes can determine the acquisition of uncontrolled cellular proliferation. As stated, telomere length serves as a control in the proliferative capacity of cells.

Neoplastic cells with telomeres shorter than their normal counterpart have been detected in many types of tumor such as neuroblastoma, tumors of endometrium, mammary tumors, lung tumors, and leukemias. In all these tumors the shorter the telomeres the more malignant was the behavior of the disease. The reason is that neoplastic cells with short telomeres have passed through many divisions, accumulating many genetic defects accounting for a more malignant phenotype. Once telomeres have reached the critic length, telomerase is activated.

As mentioned before, telomerase is normally absent in somatic cells whereas is present in germ lines to ensure the transmission of intact chromosomes to the future generations (Morin,1995). It is moreover present in hematopoietic stem cells, in T and B activated lymphocytes and in epithelial cells during regeneration. Furthermore is detectable in 90% of neoplastic tissues (Mu and Wei,2002).

Telomerase activity has not been detected, in benign or premalignant lesions such as fibrocystic mastopathy, fibroadenomas, prostatic hyperplasia, meningiomas and leiomyomas. Telomerase expression was detected, however, at the first signs of malignancy (Dashe *et al*.,1997). In these cases, the progress of telomerase activity can give information of diagnostic significance. Because normal tissues at the edges of the tumor do not express telomerase activity, it has been proposed to use telomerase activity as a marker to detect the presence of neoplastic cells at the resection margins.

Telomerase can be a useful diagnostic marker above all in mammary tumors. In one study (Hiyama *et al*., 1994) a fine needle aspiration (FNA) technique was performed in 14 patients with mammary lesions and high telomerase activity was detected. The histopathological examination confirmed the malignant nature of the lesions. In the same work a correlation between survival time and telomerase expression was noted in a group of gastric carcinomas.

The higher the expression of the enzyme, the shorter the survival time of the patient. In another work (Kim *et al*., 1994), human cells and tissue extracts were examined. Telomerase activity was detected in nine immortalized cell lines and in one ovarian carcinoma. Telomerase negative resulted four cultures of normal somatic cells and also the carcinomatous area surrounding tissue.

A correlation between telomerase expression and telomere length in a cell population has been hypothesized. To prove this hypothesis the lengths of TRFs in different immortalized cell lines used to detect telomerase activity were determined. Although the majority of them had short TRFs (< 4 kb), the rest of them had TRFs beyond 10 kb and this was enough to reject the starting hypothesis (Kim *et al*., 1994).

It has been shown that 85%-90% of human tumors are positive for telomerase activity. However, the levels of telomerase activity can vary substantially and even some normal somatic cells have been found to have low levels of enzyme activity (Hamad *et al*.,2002). It has been investigated whether there is a minimum level of telomerase activity required for tumorigenesis. Using mutants of hTERT it has been shown that there is a threshold of activity required for the process of immortalization, transformation and tumorigenesis. Thus, low level of telomerase activity detected in some somatic cells would not be expected to contribute to tumorigenesis and nor the detection of the enzyme in cancer cells necessarily signify an immortal lifespan (Hamad *et al*.,2002).

Telomerase Inhibition as an Anti-Cancer Treatment Strategy

There are a number of issues to consider in attempting to develop specific anti-telomerase therapies directed against cancer cells (Autexier and Greider,1996; Pitts and Corey,1999). First, 15% of immortal and tumor cells have no detectable telomerase activity.

Conversely, activity has been reported in some human somatic tissues (Colgin and Reddel,1999). This means that telomerase activity is not a restricted characteristic of tumor cells but can play a role in carcinogenesis along with numerous other molecular pathways. Anti-telomerase therapy could be used also in combination with chemotherapy. Another important point to keep in mind is the drug resistance issue. Targeting telomerase activity carries the risk of inadvertently inhibiting other polymerases. The development of telomerase inhibitors that manifest early secondary effects may, after all, be possible (Pitts and Corey,1999; Mata and Iversen,1997; Kondo *et al*.,1998).

Targeting the RNA Component

In mice, deletion of the telomerase RNA component resulted in the loss of telomeres and end-to-end fusion of chromosomes, but only in the 4^{th} generation of knock-out mice derived from the progenitor stock (Blasco and Greider,1997). Six to seven generations of mice were required before the mice developed abnormal phenotypes, which included defective spermatogenesis and decreased proliferation in testis and hematopoietic cells of the bone marrow and spleen (Lee *et al*.,1998). However, two telomerase inhibitors have been described that cause immediate cell death, perhaps by triggering cell cycle arrest. In yeast, deletion or mutation of the genes encoding telomerase RNA, catalytic or associated subunits of telomerase lead to telomere shortening, eventual death of most of the population and the selection of survivors able to rescue telomere lengths as a result of recombination based alternative telomere maintenance mechanisms (Colgin and Reddel,1999; Singer and Gottschling,1994; Nakamura *et al.*,1998).

Hammerhead ribozymes have also been designed to cleave human telomerase RNA (hTR) (Kanazawa *et al*.,1996). Several oligonucleotides derivatives have been tested as a telomerase inhibitors, including DNA phosphorothioate (PS) oligonucleotides, peptide nucleic

acids (PNAs) and 2'-O-methyl (2'-O-me) RNAs. An interesting class of anti hTR is constituted by the PNAs (Corey,1997). These compounds give the highest specific *in vitro* inhibition of telomerase (Norton *et al.*,1996; Hamilton *et al.*,1997; Hamilton *et al.*,1999). Compared to standard oligonucleotides or PS oligonucleotides, PNAs bind to targeted RNA more rapidly and with higher affinity and specificity. By using overlapping PNA-DNA hybrids instead of complementary ones, the authors expected to be able to use the DNA as a carrier, then allow the DNA to be digested by nucleases inside the cell. The PNAs would then be free to bind its target. None of the PNA-DNA hybrids tested were immediately toxic and the telomerase inhibition persisted for three population doublings, indicating that the amount of PNA that is delivered to the cells is sufficient to withstand the dilution caused by segregation during cellular division. After a 4 week treatment, telomere shortening was observed, confirming the specificity of the PNA inhibitors. Conclusion: the discovery that PNAs directed against non template regions of the human telomerase RNA can be potent telomerase inhibitors (Hamilton *et al.*,1999) offers a new set of targets for exploration.

Targeting the G-Quadruplex Complex

Another approach is to target the G-quadruplex, structures that can be adopted by single stranded G-rich regions such as those found at the ends of chromosomes (Wellinger and Sen,1997). It has been speculated that such structures form at chromosome ends and that these structures are essential for proper telomerase function; several molecules that bind to G quartets have been identified to inhibit telomerase activity (Sun *et al.*,1997; Izbicka *et al.*,1999; Fedoroff *et al.*,1998). However such molecule can bind both to DNA and RNA reducing their specificity (Pitts and Corey,1999) and may also inhibit processes involved in immunoglobulin gene rearrangement and transcriptional regulation (Fedoroff *et al.*,1998).

Recently, a natural product, called telomestatin, has been isolated from *Streptomyces anulatus* 3533- SV4 and it seems to have very potent anti-telomerase properties due to the structural similarity with the G tetrad. This inhibitory effect might be due to the capacity to trap out preformed G- quadruplex structures enabling telomerase to use primer molecules required for its activity (Kim *et al*.,2002).

Targeting the Catalytic Subunit

Directly targeting the catalytic protein component, hTERT instead of the RNA component, hTR, may be more effective, as telomerase activity correlates best with the expression of hTERT (Nakamura *et al*.,1997). Human telomerase reverse transcriptase is expressed in all telomerase positive cells and in only a specific subset of telomerase negative cells that are thought to have long term proliferative capacity (Kolquist *et al*.,1998). However, to date, the only reported telomerase inhibitors that targets hTERT is the expression of a mutant catalytic subunit of human telomerase (Hahn *et al*.,1999; Zhang *et al*.,1999). The effects of inhibiting hTERT by the expression of dominant negative mutants of hTERT include the inhibition of telomerase activity, telomere loss, chromosome damage, apoptosis and cell death of various human cancer cell lines (Hahn *et al*.,1999; Zhang *et al*.,1999).

Monoclonal antibodies (MAbs) capable of neutralizing hTRT represent a new strategy for use in clinical diagnosis and treatment of cancer. A recent study described the creation of an hybridoma M2 clone whose Mabs were specific for hTRT. In TRAP-PCR ELISA, M2 markedly decreased the activity of human telomerase in HeLa cells (Wang *et al*.,2002).

Telomerase Expression in Human Lymphoid Tissue

Peripheral blood lymphocytes have low to undetectable levels of telomerase expression whereas early progenitor bone marrow stem cells and thymocytes express high levels of telomerase activity. Telomerase activity is up-regulated about 1000-fold in human B lymphocytes in the germinal center. This up-regulation is thought to preserve telomere length during the extensive cellular proliferation that occurs in the response to antigenic stimulation. Telomerase can be up-regulated also *in vitro* by activation of B cells by serum immunoglobulin (sIg) or cluster of differentiation 40 (CD40) signalling. Once germinal center B cells differentiate into memory B cells, telomerase activity is down-regulated (Hu and Insel, 1999).

Peripheral blood mononuclear cells (PBMC) contain both lymphocytes and monocytes. Normal monocytes do not express telomerase activity. Thus, the low level of telomerase activity that has been found in PBMC from healthy donors represents telomerase expression in lymphocytes. Increased telomerase activity has been found both in blood lymphocytes and skin-homing T-cell lymphocytes, even in the early stages of proliferative diseases such as parapsoriasis. Higher levels of telomerase activity were found in skin-homing T-cell lymphocytes compared with that of PBMC. One possible reason could be that telomerase activity reflects proliferation of the cells or, more likely, that telomerase activity is present in a subset of malignant or pre-malignant cells (Wu *et al*., 1999). In this research study, the presence of telomerase activity and reduced telomere length in skin-homing T-lymphocytes from patients with CTCL has been supported. Similar results have been achieved in blood lymphocytes, supporting the concept that CTCL lymphocytes, both in the blood and in the skin, are predestined to become malignant T-lymphocytes.

The induction of telomerase during the activation of T lymphocytes has recently been characterized. In cultured lymphocytes, telomerase activity can be induced by exposure to phytohemoagglutinin (PHA), interleukin 2 (IL-2), anti CD3 monoclonal antibodies (MoAb), calcium ionophore A23187 and phorbol esters (Kosciolek and Rowley,1998).

Germ line cells seem to maintain a stable length of their telomeres while bone marrow stem cells and lymphocytes lose telomeric repeats despite telomerase activity both *in vivo* and *in vitro*. Germinal center (GC) B cells express high levels of telomerase activity. Significant telomere lengthening was observed as naive B cells matured to centroblasts and when centroblasts further mature to centrocytes, resulting in an increase in telomere length of about 4 kbp as determined by Southern blotting techniques. (Norrback *et al*., 2001). Along with the telomere lengthening, telomerase activity was up-regulated to high levels in the centroblasts and further more in the centrocytes. These authors speculated that this could be explained by an accumulation of telomerase with each cell division in the centroblast population, which then matures into centrocytes. Non-GC cells (resting naive, activated naive and memory B cells) have low or undetectable telomerase activity (Norrback *et al*., 2001).

Telomerase activity was detected in human lymphocytes obtained from normal donors. Telomerase was up-regulated within 24 hours when peripheral blood mononuclear cells were cultured in the presence of phytohemagglutinin. The activity increased gradually over 72 hours, than remained stable for 96 hours. These results demonstrate that telomerase is regulated during lymphocyte activation as cells progress from G0 to S phase (Yamada *et al*.,1996).

In a previous work (Broccoli *et al*., 1995), bone marrow and peripheral blood leukocytes from 19 lymphocytic leukemia patients were found to express high levels of telomerase activity. Telomerase activity was also detected in nonleukemic bone marrow and peripheral blood

leukocytes from normal donors. These data indicated that human telomerase is not restricted to the immortal phenotype. Telomerase expression was not expected in human hematopoietic cells because both circulating and bone marrow leukocytes show losses of telomeric repeats over time. One explanation is that telomere shortening can occur despite telomerase activity. Based on this work (Broccoli *et al*.,1995) telomerase activity per se may be not directly correlated to disease in all malignant tumors. In another study (Norrback *et al*.,1996), strong telomerase activity was demonstrated *in vivo* in normal mature lymphocytes, as well as in malignant lymphoma. Benign lymph nodes had lower telomerase activity compared with the malignant counterpart. High-grade lymphomas had higher levels of telomerase activity compared with low-grade lymphoma. The data showed that *in vivo* activation of telomerase is a characteristic feature of germinal center B cells. Different signal for activation of telomerase are likely to exist, and one of them can be immune stimulation. The authors suggest an "induction and retention" model to explain telomerase activity in lymphomas. Based on this model, transformation occurs in a normal, mature B cell with reactivated telomerase, which is retained in the neoplastic clone (Norrback *et al*.,1996). High telomerase activity was also detected in non-Hodgkin's lymphomas (Lin *et al*., 2001). Enhanced telomerase activity combined with deregulation of the factors responsible for cell survival and proliferation may contribute to the development and progression of lymphomas (MacNamara *et al*.,2001).

Telomerase activity was detected in skin biopsies from four cutaneous T-cell lymphoma (CTCL) or mycosis fungoides (MF) patients. From these biopsies, cell lines have been established. Both skin-homing T-cell lines and peripheral blood mononuclear cells (PBMC) from patients with MF had high telomerase activity and short telomere length, whereas a low level of

telomerase expression and normal telomere length was found in patients with lymphomatoid papulosis and among healthy donors (Wu *et al*., 1999).

In a different study it has been investigated whether telomerase expression was useful for the detection of occult malignant cells in lymph nodes (Yashima *et al*.,1997). The TRAP assay was used in this research project. The results of the study indicated that 96% of the histologically negative nodes expressed low levels of activity that may be derived from activated lymphocytes that express telomerase activity. All the malignant nodes expressed telomerase. *In situ* results showed clearly that the hTR was expressed relatively highly in metastatic cancer cells and at low levels in germinal centres of secondary follicles. The conclusion of the work stated that although telomerase expression by activated lymphocytes may limit its usefulness, measurement of enzyme activity combined with detection of hTR using in situ hybridization may assist in the histopathological diagnosis of lymph nodes (Yashima *et al*.,1997).

Telomerase Activity in Canine Tissues

The canine species has been proposed as an alternative model system to study telomere and telomerase biology (Nasir *et al*.,2001). The TRAP Assay has been shown to be suitable for canine tissue (Biller *et al*.,1998).

One of the first works that focused on the evaluation of telomerase activity in neoplastic tissue of dogs assessed 33 client-owned dogs with different kinds of neoplastic diseases diagnosed by histopathology. Oral, thoracic and abdominal masses as well as skeletal muscle and bone lesions were the most representative samples in this study. Twenty-four of 26 malignant tissues, 1 of 4 benign tumors and 0 of 3 normal tissues were telomerase positive (Biller *et al*.,1998).

In a different study (Yazawa *et al*.,1999) telomerase activity was measured using the TRAP assay. Normal dog somatic tissues showed little or no telomerase activity, while normal testis exhibited a high level of telomerase activity. Telomerase activity was measured in tumor samples from 45 dogs: 21 mammary gland tumors; 16 tumors of the skin and oral cavity; 7 vascular tumors; and 1 Sertoli cell tumor. Greater than 95% of the tumor samples tested had detectable telomerase activity.

The same authors, subsequently evaluated canine mammary tumors. Twenty-seven mammary gland tumor specimens obtained during necropsy and 12 mammary gland tissue specimens obtained from healthy dogs were examined for telomerase activity. Telomerase activity was detected in 26 of 27 mammary gland tumors and in 4 of 12 normal tissue specimens (Yazawa *et al*.,2001).

Recently telomerase activity was evaluated in lymph nodes, buffy coat, and serum samples from dogs with malignant lymphoma and in liver, lymph node, buffy coat and serum samples from clinically normal dogs (Carioto *et al*.,2001). The TRAP assay was the method utilized in this experiment. Of 11 clinically normal dogs, 8 had lymph node samples, 5 had liver samples and 1 had buffy coat samples with detectable telomerase activity. None of the serum samples from the clinically normal dogs had detectable telomerase activity. Of 14 dogs with lymphoma, 9 had lymph node samples, 3 had buffy coat samples, and 1 had serum samples with measurable telomerase activity. These authors concluded that telomerase activity was not specific for tumor cells in the dog. There was no difference in telomerase activity in lymph nodes of clinically normal dogs and dogs with lymphoma.

The presence or absence of telomerase activity in canine and feline body cavity effusions was compared with the cytologic evaluation (Spangler *et al*., 2000). Detection of telomerase in

effusions was no more sensitive than cytologic evaluation for the identification of neoplasia. In this study cytologic evaluation was more specific than the TRAP assay (telomerase assay: sensitivity = 50%, specificity = 83%; cytology: sensitivity = 50%, specificity = 100%). The conclusion of this work pointed out the telomerase assay may constitute a useful adjunctive test for the diagnosis of neoplastic disease in dogs and cats with body cavity effusions. However, the results of this assay are not sufficiently reliable to be used as a sole diagnostic test (Spangler *et al*., 2000).

Another study hypothesized that telomerase activity was present in both normal and lymphoma lymph nodes and that neoplastic tissue had higher level of telomerase activity (Hipple *et al*.,2001). Statistical analysis found no difference in telomerase activity between normal and lymphoma lymph nodes. This study concluded stating that telomerase activity was not predictive of survival or remission duration in dogs with lymphoma.

Lymphoma in the Dog

Epidemiology

Lymphoma is a neoplasm of malignant lymphocytes. Lymphoma arises from a clonal expansion of lymphoid cells with distinctive morphologic and immunophenotypic features. Lymphoma is one of the most common malignant tumors in the dog and it is the most common hematopoietic tumor in this species. Seven to 24% of all canine neoplasias are lymphomas. Lymphoma comprises 83% of all hematopoietic tumors in dogs. Middle-aged to older dogs (6-9 years) are primarily affected (Withrow and MacEwen, 2001). Breed predisposition has been reported for boxers, Scottish terriers, basset hounds, Airedale terriers, chow-chow, German shepherds, poodles, St. Bernards, English bulldogs, beagles, and golden retrievers (Keller *et al*., 1993).

Clinical Presentation

The typical anatomic sites for canine lymphoma are multicentric, alimentary, mediastinal (thymic) and extranodal (renal, CNS, skin, eyes). The multicentric form is the most common accounting for up to 84% of all cases. Alimentary lymphoma is the second most common (7%) anatomic form followed by the extranodal form (< 7%) and the mediastinal form (< 2%) (Morrison WB,1998).

Multicentric Lymphoma

Multicentric lymphoma involves multiple lymph nodes and may also extend to liver, spleen, and bone marrow (BM). Dogs with multicentric lymphoma are usually middle-aged and are presented for painless, recently noticed lymphadenopathy. Nonspecific clinical signs include weakness, lethargy, vomiting and weight loss. Ten to 20% of dogs with multicentric lymphoma have hypercalcemia of malignancy (Morrison WB., 1998). Approximately 60% to 75% of dogs with multicentric lymphoma have abnormalities on thoracic radiographs including interstitial pulmonary infiltrates and thoracic lymphadenomegaly. Abdominal radiographs display evidence of sublumbar lymph nodes, spleen or liver involvement in 50% of cases (Starrak *et al*.,1997; Blackwood *et al*.,1997).

Alimentary Lymphoma

Alimentary lymphoma may be characterized by a single mass or may have a diffuse intestinal involvement. Clinical signs associated with the alimentary form of lymphoma include weight loss, vomiting, diarrhea, anorexia, malabsorption and melena.

Mediastinal Lymphoma

The mediastinal form involves the cranial mediastinal lymph nodes or the thymus. Dyspnea, coughing, regurgitation, exercise intolerance and dysphagia are clinical signs

associated with mediastinal lymphoma. Compression of the vena cava can cause cervical and facial edema. Up to 40% of dogs with mediastinal lymphoma present with hypercalcemia (Morrison WB., 1998). Pleural effusion may be seen. The mediastinal form of lymphoma in dogs is most commonly associated with a T-cell phenotype (Ruslander *et al*.,1997).

Extranodal Lymphoma

Extranodal lymphoma involves all non-lymphoid organ sites, such as central nervous system (CNS), skin, kidney, eyes and heart. Clinical signs associated with the extranodal form may be nonspecific (anorexia, weight loss, lethargy, PU/PD) or specific depending on the organ involved. Lymph nodes involvement may be or may be not present.

Clinical Pathology

Laboratory abnormalities, seen in dogs with lymphoma, include anemia (38% of cases), thrombocytopenia (58% of cases) caused by bone marrow invasion or immune complications (Madewell BR,1986), or hypercalcemia (10-40% of cases) secondary to the production of a parathyroid hormone-related peptide (PTHrP) (Teske E, 1994). Specific organ involvement may be reflected in lab work.

WHO Staging of Lymphoma

Canine lymphoma is clinically staged in five categories according to the involvement of specific anatomic compartments or organs (**Table 5**). Each of the five stages may include two different sub-stages. Substage A is considered any stage without systemic signs of disease whereas substage B is considered any stage with systemic signs of disease.

Diagnosis

Several examinations are available to help the clinician in the diagnostic process of lymphoma. A complete physical examination, hematologic panel, serum biochemistry panel, fine needle aspiration (FNA), radiographic and ultrasonographic imaging, histopathology and ophthalmologic examination should be performed. While cytology provides a diagnosis of lymphoma, a tissue biopsy should be performed to confirm the diagnosis and to histologically grade the tumor. Immunophenotyping by immunohistochemistry or immunocytochemistry is becoming more available to veterinary clinicians (Carter RF and Valli VEO., 1988). Thoracic radiography may reveal abnormalities including enlargement of the retrosternal or tracheobronchial lymph nodes, pulmonary infiltrates, mediastinal masses, and pleural effusion. Abdominal radiographs may show alteration in organ size and structure. Laboratory examination may reveal hematological and serum abnormalities such as anemia, thrombocytopenia, leukopenia and hypercalcemia. Peripheral blood smears, bone marrow aspirate and bone marrow core biopsy may be helpful in the staging process.

Prognostic Factors

Several prognostic factors have been established for canine lymphoma. Negative factors include high stage (stage V), substage B, hypercalcemia, mediastinal lymph node involvement, T-cell phenotype, lack of response to chemotherapy, histopathological grade and p-glycoprotein expression (Morrison WB.,1998).

Treatment and Prognosis

The therapeutic approach to a patient with lymphoma is determined by the stage or substage of disease, the presence of paraneoplastic syndromes, intercurrent disease status, and

financial concerns of the client. Without treatment, most dogs die within 4 to 6 weeks of diagnosis (Withrow and MacEwen., 2001). Lymphoma is a very responsive tumor to chemotherapy. Conventional chemotherapy results in approximately 60% to 90% of dogs achieving complete response (CR), with median survival duration of 6 to 12 months, depending upon the chemotherapy protocol employed (Withrow and MacEwen., 2001). Noticeable remission may begin within a few hours of drug administration, but typically occurs within the first week of treatment. Most first remissions last 6-8 months. Second and subsequent remissions are increasingly more difficult to obtain and are of briefer duration than first remission. Overall survival time for dogs with lymphoma is generally 9-12 months. The primary goal of chemotherapy is to induce a complete and durable remission, then to re-induce or rescue a remission after one or more relapses. Most relapses are due to inadequate dosing and frequency of administration, development of multidrug resistance (MDR) following exposure to selected chemotherapeutic drugs (Kartner *et al*.,1983; Bergman *et al*.,1996) and failure to achieve sufficient concentrations of drugs in specific privileged anatomic locations such as the brain.

There are many chemotherapeutic protocols available to treat lymphoma in dogs. Single agent chemotherapy or multidrug protocols are chosen according to individual case and factors.

Single agent therapy is not considered the standard therapy for canine lymphoma. However it represents the simplest and cheapest option. With corticosteroids most dogs attain a short remission and improve quality of life. Median survival time for dogs treated with steriods alone is 53 days compared with untreated dogs that live 30 days on average. It has been shown that steroids therapy can induce the phenomenon of drug resistance by overexpressing p-glycoprotein, a cell membrane pump required for removal of drugs from cells. Cyclophosphamide is an alkylating agent commonly used in the treatment of lymphoma. Dogs

treated with cyclophosphamide alone can achieve a longer remission duration (median 165 days) compared with prednisone. Shorter remission duration have been noted for chlorambucil alone or even in combination with prednisone compared with cyclophosphamide. Chlorambucil is used as a substitute for cyclophosphamide in the case of sterile hemorrhagic cystitis. Most dogs show a partial response rate to L-asparaginase single agent. Vincristine and vinblastine can produce a short remission time when used alone (30-180 days). Around 56% of dogs with lymphoma treated with single agent doxorubicin obtained a complete or partial remission. The median duration of the first remission was 38 days and median survival time was 100 days. This drug has the potential for considerable side effects including anaphylaxis, perivascular sloughing, myelosuppression, gastrointestinal and cardiac toxicities. Epirubicin is used as a substitute of doxorubicin. Mitoxantrone is a minimally cardiotoxic analog of doxorubicin. Actinomycin D is another anti-tumor antibiotic used in the treatment of lymphoma. Lomustine (CCNU) is an alkylating agent that has been used as a rescue agent for refractory multicentric lymphoma (Morrison,1998).

Most of multi-drug protocols have induction and maintenance phase. Many of the protocols used in dogs are based on the combination of cyclophosphamide, vincristine and prednisone, with or without doxorubicin. The COP protocol consist of cyclophosphamide, vincristine and prednisone given for 6 weeks of induction, followed by a maintenance protocol of methotrexate, higher dose cyclophosphamide, and lower dose prednisone for an additional 6 weeks. Complete remission and partial remission were achieved in 69% of the dogs. Median survival time was 123 days. The CHOP protocol contains the same drugs contained in the COP plus doxorubicin. Median survival time for this protocol is about one year. The University of Wisconsin-Madison protocol (vincristine, L-asparaginase, cyclophosphamide, prednisone and

doxorubicin) is reported to have one of the longest remission (252 days) and survival time (357 days). A different protocol is known as COPLA (induction phase with cyclophosphamide, vincristine, prednisone, L-asparaginase and doxorubicin, and maintenance phase with vincristine and chlorambucil). Remission and survival times are the same as most combination protocols, but the number of dogs achieving remission is high and the protocol is well-tolerated (Morrison,1998). The most common protocols for refractory lymphoma are those including doxorubicin/dacarbazine combination and the MOPP protocol (methclorethamine, vincristine, procarbazine, and prednisone). Usually a 40% to 50% response rate is achieved (Withrow,2001).

CHAPTER 3. TELOMERASE ACTIVITY IN CANINE LYMPHOCYTES

Objectives

Based on the hypothesis that telomerase is a specific marker for neoplastic tissues, the development of methods to detect its activity may represent an accurate, non-invasive diagnostic and prognostic tool for use in clinical veterinary medicine. The aim of this study was to detect telomerase activity in normal, hyperplastic and neoplastic lymphocytes comparing two different detection methods: the TRAP assay and the TRAP ELISA Telomerase Detection Kit. These tests are commercially available and commonly used to detect telomerase activity. The TRAP assay is a PCR based protocol that relies on detecting a ladder of fragments after electrophoresis of a polyacrylamide gel. The TRAP ELISA utilizes a colorimetric detection method, omitting the gel preparation step. The TRAP ELISA seems to be easier and less time consuming to perform. This study was undertaken to establish the relative sensitivity and specificity of these methods in canine lymphoid tissues.

CHAPTER 4. MATERIALS AND METHODS

Study Population

The study population consisted of client-owned dogs that underwent surgical biopsy or exploratory procedure during their hospitalization at the University of Illinois – Veterinary Teaching Hospital (VTH). Tissue sample in excess of that required for clinical diagnosis were collected from the operating room. Informed consent was given by the owner for collection of part of the samples used for this study. This study population included dogs with neoplastic or non-neoplastic conditions and all dogs with neoplastic diseases had a diagnosis of lymphoma. Normal lymph nodes samples were harvested from control research dogs euthanized as part of other research studies.

Sample Collection and Storage

Resected tissue was submitted for diagnostic histopathology analysis in formaldehyde preservative to a board certified veterinary pathologist at the University of Illinois Veterinary Diagnostic Laboratory. At surgical biopsy, part of each sample was rapidly frozen in liquid nitrogen, then stored at -80°C until used for the molecular procedures described herein.

Telomerase Extract Preparation

Tissue extracts were obtained from frozen samples by mincing 40-100 mg of tissue with a sterile scalpel blade. Samples were transferred to a sterile 1.5 ml microcentrifuge tube and 200 µl of 1X ice-cold CHAPS lysis buffer [10 mM tris-HCl (pH 7.5), 1 mM $MgCl_2$, 1 mM EGTA, 0.1 mM Benzamidine, 5 mM β-mercaptoethanol, 0.5% CHAPS and 10% Glycerol] was added.

At this point, homogenization with a motorized pestle was performed until a uniform consistency was achieved (~ 10 seconds). After, that the suspension was incubated on ice for 30 minutes, centrifuged at 12,000 x g for 20 minutes at 4˚C. Each supernatant (approximate volume 160 µl) was transferred into a fresh tube and stored at - 80˚C. Protein concentration was determined for each extract using the Coomassie protein assay reagents[1].

TRAP Assay

Principle and Methodology of the Assay

In this experiment, the Intergen's TRAPeze® Telomerase Detection Kit was used. This kit is based on an improved version of the original method described by Kim, et al, which is more accurate and rapid to perform. The kit is a highly sensitive *in vitro* assay for detecting telomerase activity. In the first step of the reaction, telomerase adds a number of telomeric repeats (GGTTAG) onto the 3' end of a substrate oligonucleotide (called the TS primer). In the second step, the extended products are amplified by PCR using the TS and RP (reverse) primers to generate a ladder of products in 6 base pair increments starting at 50 nucleotides. This product is visualized on a 12% polyacrylamide gel.

Experimental Control Included in Each Assay

Heat inactivation control. Because telomerase is a heat sensitive ribonucleoprotein enzyme, every sample extract should also be tested for heat sensitivity as a negative control. Thus, analysis of each sample consists of two assays: one with a test extract and one with a heat-treated test extract. Heat inactivation was achieved by incubating the sample at 85˚C for 15 minutes prior to the TRAPeze kit amplification.

[1] Pierce, Rockford, IL.

Telomerase positive control. The TRAPeze Kit includes a known telomerase positive cell pellet. An extract was made from the pellet as described for tissue extracts and included as a positive standard control in each TRAP assay. In addition we included a cell pellet created in our laboratory from a known telomerase positive canine osteosarcoma line.

PCR amplification control. Two internal control oligonucleotides called K1 and TSK1 were included in each reaction mixture. When paired with the TS primer, provided in the kit, they produce a 36 base pair (bp) band (S-IC) in every lane on the gel. Visualization of this band allows verification of PCR amplification and elimination of false-negative results. False negative can result from the presence of inhibitors of *Taq* polymerase sometimes contained in the cell or tissue extracts.

Primer-dimer/PCR contamination control. Non-specific primer annealing produce PCR artifacts that occur by introduction of primers alone without appropriate template DNA. This binding pattern represents a negative control for the assay.

TSR8 quantitation control. TSR8 is an oligonucleotide with a sequence identical to the TS primer but extended by 8 telomeric repeat hexanucleotide sequences, $AG(GGTTAG)_7$. When TSR8 is included as the template DNA rather than the sample extract this serves as a second positive control and as a standard for estimating the amount of TS primers extended by telomerase in a given sample extract.

TRAP Assay Reactions

One µl of each extract was added to 24 µl of the Master mix for a total volume of 25 µl. The tubes were then placed in the thermocycler block and incubate at 30ºC for 30 minutes. We used a modified version of the PCR program provided with the kit because prior work in our laboratory allowed us to optimize the assay for use in canine tissues.

Cycle conditions consisted of a melting phase, an annealing phase and a final extension-amplification phase.

94ºC x 30"
50ºC x 30" } x 30
72ºC x 30"
70ºC x 10'

Gel Electrophoresis

Five µl of gel loading dye containing bromophenol blue and xylene cyanol was added to each sample tube after PCR amplification. Approximately 28 µl of each sample was loaded into appropriate lanes of a 12% non-denaturing polyacrylamide gel. To prevent contamination between samples in adjacent gel lanes, alternate lanes were loaded. The gel was run in 1X Tris-Boric acid-EDTA buffer (TBE) in a vertical gel box for 3 hours at 34 mA.

Visualization

After electrophoresis, the gel was stained with SYBER Green[2] diluted approximately 1:10,000 in 1X TBE. The gel was than shaken gently on a rocker platform for at least 15 minutes in the stain solution. An ultra-violet light box was used for band detection. Results were captured with a Polaroid camera system.

TRAP-ELISA Assay

Principle and Methodology of the Assay

In this experiment, Intergen's TRAPeze® ELISA Telomerase detection kit was used. The TRAP-ELISA method is based on an alteration of the original assay described by Kim et al. The kit includes reagents for non-radioactive detection of the telomerase products by an ELISA

[2] Roche, Indianapolis, IN.

protocol. The procedures are separated into two steps: TRAP extension/amplification and detection. In the first step of the reaction, telomerase adds a number of telomeric repeats (GGTTAG) onto the 3' end of a biotinylated Telomerase Substrate oligonucleotide (b-TS). In the second step, the extended products are amplified by the polymerase chain reaction (PCR) using Taq polymerase, the b-TS primer and RP (reverse) primers, and a deoxynucleotide mix containing dCTP labeled with dinitrophenyl (DNP). This extension/amplification reaction generates a ladder of products with 6 base increments starting at 50 nucleotides. Thus, the TRAP products are tagged with biotin and DNP residues. The labeled products are immobilized onto streptavidin-coated microtiter plates via biotin-streptavidin interaction and then detected by anti-DNP antibody conjugated to horseradish peroxidase (HRP). The amount of TRAP product is estimated by means of the HRP activity using a colorimetric substrate 3,3',5,5'-tetramethylbenzidine (TMB). While the method is not-completely quantitative, telomerase activity in extracts made from 30-1,000 telomerase positive cells can readily be measured. Telomerase activity from as few as 30 telomerase positive cells among 3,000 negative cells can be detected with the TRAPeze® ELISA.

The experimental controls included in this assay were the same used for the TRAP assay procedure.

TRAP ELISA Assay Reactions

Two µl of each extract were added to 48 µl of the Master mix for a total volume of 50 µl. The tubes were then placed in the thermocycler block and incubate at 30ºC for 30 minutes. We used a modified version of the PCR program provided with the kit, because prior work in our laboratory allowed us to optimize the assay for use in canine tissues: 94°C/30 sec., 55°C/30 sec. for 33 cycles.

ELISA Detection

Pretreatment of the microtiter plate (MPT) consisted of the addition of 250 µl of blocking/dilution buffer into each well, incubation at 37°C for 30 minutes, removal of the blocking buffer and rinsing with 1X washing buffer. Subsequently, 100 µl of the blocking buffer were added to each well along with 5 µl of the TRAP reaction product created. After 1 hour incubation at 37°C, the solution was removed and wells rinsed 5 times with 1X washing buffer. In the meantime, the stock antibody solution was diluted 1:250 with blocking buffer and 100 µl of anti-DNP antibody-HRP conjugate were added to each well. After 30 minutes incubation at room temperature the antibody solution was removed and wells rinsed 5 times with 1X washing buffer. Subsequently, 100 µl of TMB solution were added to each well and the mix was incubated for 10 minutes at room temperature. Finally, 100 µl of stop solution were added to each well. This last reaction causes color change of the peroxidase product from blue to yellow. The absorbance of the samples was measured at 450 and 630 nm.

Data Analysis

In order for the TRAP ELISA assay to be valid, the controls must have the following absorbance measurements: primer/dimer/PCR contamination control should have a value of absorbance (A) < 0.200; TSR8 PCR/ELISA control should have a value > 0.800; heat inactivated sample extract should have a value < 0.250; experimental samples should have a value $\Delta A > 0.150$ to be positive ($\Delta A = A_{sample} - A_{heat\ treated\ sample}$).

CHAPTER 5. RESULTS

TRAP Assay Results

The TRAP Assay was used to examine neoplastic and reactive lymphoid tissues of client-owned dogs. Normal node tissues were obtained from control dogs euthanized at the conclusion of unrelated projects. Fifteen lymphomatous tissue samples, 13 normal lymph nodes and 2 reactive lymph nodes were available for the study. Telomerase positive samples were characterized by a relatively intense band at 50 base pairs with a ladder of incrementing bands at intervals of 6 base pairs. The comparator for this study was the histopathological examination. Based on this method we obtained a very strong correlation between the histopathologic diagnosis and the telomerase outcome. Of 15 histopathologic diagnosed lymphomas, 12 were telomerase positive (80%) and 3 were telomerase negative (20%). Of 15 non-lymphomatous nodes, including normal and reactive nodes, 14 were telomerase negative (93%) and only one was telomerase positive (6.7%). Both of the two reactive lymph nodes were telomerase negative. Analysis of these results indicates a sensitivity of 80% and specificity of 93% for this method (**Table 7**).

TRAP ELISA Results

This method was used simultaneously using the same samples tested by the TRAP assay. Of 15 lymphoma samples tested, 11 were positive (73%) whereas 4 were negative (27%). Of 15 non-lymphomatous nodes, including normal and reactive nodes, histologically normal lymph nodes, 2 were telomerase positive (15%), whereas 13 were negative (85%). Both of the two

reactive lymph nodes were telomerase negative. Analysis of these results indicates a sensitivity of 73% and specificity of 87% for this method (**Table 8-9**).

Statistical Analysis

Sensitivity, Specificity and Beta Distributions for TRAP Assay (**Table 7, Fig. 1-2**):

Sensitivity = a/a+c = 12/(12+3) = 80%

Specificity = d/b+d = 14/(1+14) = 93%

Sensitivity, Specificity and Beta Distributions for TRAP ELISA (**Table 8, Fig 3-4**):

Sensitivity = 73%

Specificity = 86%

Diagnostic Odds= 17.8

In summary, we can say that estimates of sensitivity and specificity inherently are uncertain and the estimate of potential error is measured by the β-distribution of the sample population. When the TRAP ELISA test is applied to a specific dog we cannot know which is the actual value of sensitivity and specificity, but we know that all the possible values are those described by the β-distribution (**Table 10-11**). By means of the simulation, we have virtually applied the test to a sample of 1000 dogs with an 80% probability that the dog in question has lymphoma. Analysis of 1000 virtual samples allows the simulation to calculate the final probability value for a dog to have a tumor after a positive outcome on the TRAP ELISA. The computer obtained this result by combining all the possible values of sensitivity and specificity described by the β-distribution. The computer also considered the probability associated with the

single value, that is, the probability that a given different value can be registered. This analysis states that if we obtain a positive result using 1000 dogs for the TRAP ELISA: 25% of dogs will have a probability to truly bear a tumor ranging from 80% and 92%; 25% of the dogs will range between 92% and 94%; 25% will range between 94% and 96% and the last 25% will range between 96% and 100%. Based on this we can state that the TRAP ELISA is truly predictive of the dog's disease status.

CHAPTER 6. DISCUSSION

In this study the TRAP and the TRAP ELISA assay have been used to detect telomerase activity in normal and neoplastic canine lymph nodes. Our results indicate a strong correlation between telomerase activity and presence of lymphoma in the animal by using both of these two methods. |Compared to the conventional TRAP assay, the TRAP ELISA is less time consuming because neither gel preparation nor electrophoretic separation steps are required. Telomerase activity from as few as 30 telomerase positive cells among 3,000 negative cells can be detected with the TRAPeze® ELISA.

While the manufacturer describes the TRAP ELISA assay to be more sensitive and specific than the TRAP assay, we found that the TRAP assay was more sensitive in this application.

The value of detecting telomerase activity is that this activity has potential clinical application as a diagnostic, prognostic and therapeutic tool. However, it is important to understand that while 90% of human tumors are positive for telomerase activity, the level of activity can vary substantially. Also, selected normal somatic cells have been found to have low levels of enzyme activity (Hamad *et al*., 2002). Thus, low levels of telomerase activity detected in some somatic cells should not be considered an indication for tumorigenic potential and nor should the detection of the enzyme in neoplastic cells necessarily signify an immortal phenotype (Hamad *et al*., 2002).

In the case of lymphatic tissues, human peripheral blood lymphocytes have low to undetectable levels of telomerase activity (Hu and Insel, 1999) whereas bone marrow progenitor stem cells and thymocytes express high levels of enzyme activity. Different studies indicate

increased telomerase activity in both blood lymphocytes and skin-homing T-cell lymphocytes (Wu *et al*., 1999). In germinal center B lymphocytes, telomerase expression is up-regulated about 1000-fold as compared to cells not located within the germinal center. Significant telomerase up-regulation was observed to occur as naïve B cells matured to centroblasts and further in centrocytes. This up-regulation is thought to be required for preserving telomere length during the extensive clonal cellular proliferation that follows antigenic stimulation. Once germinal center B lymphocytes differentiate into memory B cells, telomerase activity is down-regulated. These studies indicate the importance of cellular developmental and replicative status to the interpretation of telomerase activity. Based on these and other results, telomerase activity per se may be not directly correlated to malignancy in all situations (Broccoli *et al*., 1995).

An important advance to improve interpretation of telomerase activity as a predictor of patient status is quantitation of the enzyme activity. Most of the methods commonly used to detect telomerase activity are not quantitative. The TRAP and the TRAP ELISA assays cannot quantify the actual level of telomerase within the examined samples, due to the variability inherent in PCR amplification method. This limitation is much more important when examining lymphoid tissue because telomerase may be present in the malignant and in the benign cells of a tissue, but expressed at different levels. A key goal is to determine a "threshold" in terms of quantitation, to differentiate physiologic from pathologic telomerase expression.

Assessment of the banding pattern created by using the TRAP assay requires some level of subjective interpretation. The band pattern may not be clear, leading to misinterpretation of samples with low levels of activity as false negative results. False positive results are much less likely with the TRAP assay. The TRAP ELISA detection method allows less subjectivity in interpretation because specific absorbance ranges must be detected in order for a sample to be

declared positive. Perhaps the best way to eliminate subjective interpretations would be to use one of the newly described quantitative methods such as the new ELIPA and RTQ-TRAP assay. The precise quantitation of telomerase activity could provide the clinician with more accurate information than the current "positive" and "negative" results generated with both the TRAP and the TRAP ELISA assays.

Potential reasons for false negative telomerase results in histologically diagnosed lymphoma, include RNA degradation during sample collection, storage or processing. Because of the fragility of the RNA component of this ribonucleoprotein molecule, it is very important that the investigator be accurate and sterile in all the steps of the procedure. Sample proper storage conditions are also essential. Detection of amplification of the K1 and TSK1 control primers helps eliminate false negative results caused by Taq polymerase inhibitor substances present during PCR amplification. These control primers do not control for inactivation of the telomerase enzyme itself, however, false negative results may be seen because samples with low levels of telomerase activity may be below the limits of detection for the assay utilized. It is also important to mention that true telomerase negative tumors have been reported. A possible explanation for this phenomenon could be that telomerase may require a certain period of time (population doubling "lag") before being activated in neoplastic cells. The period of time required for activation of the enzyme may be strictly related to original telomere length of the affected cells. Based on this concept, tumors with long telomeres might not require telomerase activation, or might recruit up-regulation or down-regulation of other specific genes to gain immortality and endless proliferative capacity.

Telomerase positive results from normal lymph node samples represent another concern to be considered when using the assay as a diagnostic test for lymphoma. Normal germinal

center B lymphocytes have been shown to express telomerase activity after antigenic stimulation. Based on this phenomenon, samples from normal lymph nodes containing many germinal centers have a greater probability of being detected to express telomerase activity without any correlation to neoplasia. Because germinal center B cells are typically telomerase positive, we expected samples from reactive nodes to be positive. However, only two reactive nodes were available for this study and both were considered to be telomerase negative. Obviously this represented too small a sample size to make any meaningful conclusions regarding telomerase activity in reactive lymph nodes. Another concern to be considered when dealing with potential telomerase false positive results is the potential for PCR contamination with telomerase enzyme from sources other than the tissue being tested.

In veterinary medicine, there is controversy among the different studies conducted to determine telomerase activity in normal and neoplastic tissues. A strong correlation between telomerase expression and malignancy was reported by several investigators (Biller *et al*., 1998; Yazawa *et al*., 1999; Yazawa *et al*., 2001). According to Carioto's work (Carioto *et al*., 2001) telomerase activity is not specific for tumor cells in dogs with lymphoma. Detectable telomerase activity was found in lymph node samples from clinical normal and from lymphoma-bearing dogs. This lack of discrimination was also observed in Hipple's study (Hipple *et al*., 2001).

A clarifying step to better resolve this controversy would be to establish which histologic aspect of the normal lymph node was submitted for telomerase examination. By knowing whether or not the examined samples contains germinal centers, it would be possible to better interpret a positive or negative test result. Based on the study reported from human medicine, this quality assurance step aids in resolving this problem. In all telomerase studies reported in veterinary medicine, including those conducted in our laboratory, this clarifying step has not

been done. This omission leaves a source of uncertainty that could be resolved in future investigation. Another issue to consider is that we did not classify our samples according to the anatomical site from which the lymph node was harvested. Theoretically, mesenteric and peripheral lymph nodes could present different expression of telomerase activity based on the different levels of antigenic stimulation to which they are subjected. Knowing the histologic grade of the disease could prove very helpful in interpreting telomerase results as well. Negative results from histologically confirmed low-grade lymphoma could be explained by assuming that telomeres in cell of this type of disease are not subject to as much erosion due to cell replication as seen in high grade disease. Alternately, low-grade lymphoma may have cells with telomeres long enough to prevent telomerase activation. In our study, two of our lymphoma samples were negative for telomerase expression by both procedures. The histopathological assessment of these samples reported that one samples was diagnosed as low-grade lymphoma (sample # 10) and the other sample was "suspicious of lymphoma" (sample # 15) (**Table 6**).

In the present study we found a strong correlation between telomerase expression and the biological status of the animal with regard to malignancy. One of the greatest weakness in all reported veterinary studies, including our own, is the lack of statistically significant sample size evaluated in most reports. We attempted to partially overcome this limitation by utilizing the Monte Carlo simulation method. With this simulation, we assessed 1000 "virtual" dogs with an 80% pre-test probability of having lymphoma. These virtual dogs were assessed for telomerase activity using the sensitivity and specificity parameters determined by the TRAP ELISA assay conducted here. This allowed us to obtain a more precise estimate of the probability for the sample examined to really bear a lymphoma based on all the possible values of sensitivity and specificity determined by the beta-distribution for the samples tested in this work.

In conclusion, based on our study, we conclude that the TRAP assay is more reliable in term of sensitivity when compared with the TRAP ELISA. Telomerase analysis may be potentially useful in veterinary medicine as a diagnostic and prognostic tool for animals with lymphatic disease. Obviously we do not recommend utilizing the TRAP as the sole single diagnostic method because there remains some concern about the subjectivity of the procedure and the veracity of some of the positive and negative results. Our recommendation is to use telomerase analysis as a further confirmation after clinical, cytological and histopathological analysis.

CHAPTER 7. TABLES

Table 1. Telomeric Repeat Sequences in Eukaryotes

Group	**Organism**	**Telomeric repeat (5' to 3')**
Vertebrates	Human, Mouse, Xenopus	TTAGGG
Filamentous fungi	Neurospora	TTAGGG
Slime molds	Physarum, Didymium, Dyctiostelum	TTAGGG AG (1-8)
Kinetoplastic protozoa	Trypanosoma, Glaucoma Paramecium Oxytricha, Stylonychia, Euplotes	TTGGGG TTGGG (T/G) TTTTGGGG
Sporozoite protozoa	Plasmodium	TTAGGG (T/C)
Higher plants	Arabidopsis	TTTAGGG
Insects	Bombyx mori	TTAGG
Nematodes	Ascaris lumbricoides	TTAGGC
Alga	Chlamydomonas	TTTTAGGG
Fission yeasts	Schizosaccharomyces pombe	TTAC (A) (C) G (1-8)
Budding yeasts	Saccharomyces cerevisiae	TGTGGGTGTGGTG (from RNA template)

Table 2. The molecular cascade involved in the M1 phase entrance.

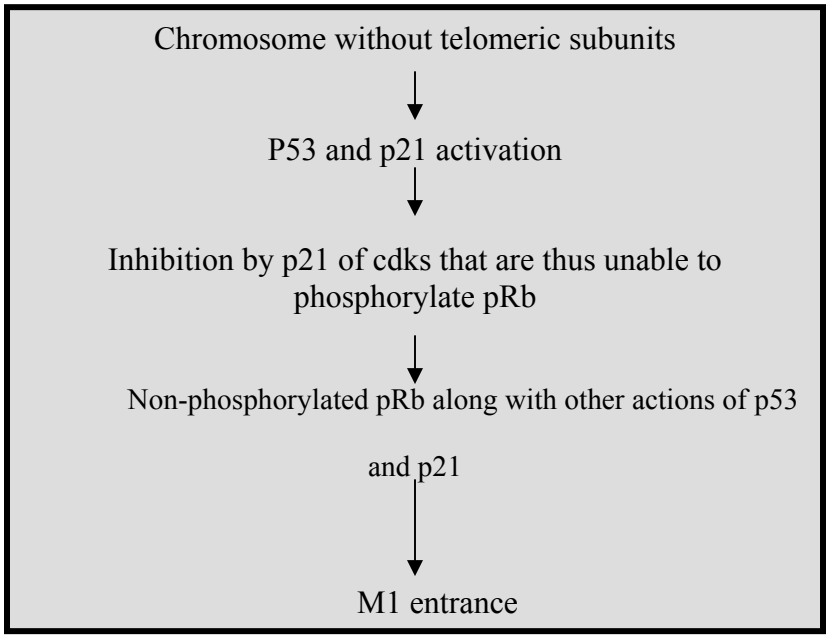


Table 3: Summary of TRAP Assay results for canine lymphoid tissue samples

Sample Category	# Tested	# Telomerase Positive (%)	# Telomerase negative (%)
Lymphoma	15	80.0%	20.0%
Normal	15	6.7%	93.3%

Table 4: Summary of TRAP ELISA Assay results for canine lymphoid tissue samples

Sample Category	# Tested	# Telomerase Positive (%)	# Telomerase negative (%)
Lymphoma	15	73.0%	27.0%
Normal	15	13.3%	86.7%

Table 5. Clinical staging for canine lymphoma

Stage	
I	Involvement of a single lymph node or lymphoid tissue in a single organ
II	Involvement of regional lymph nodes with or without extra-nodal site
III	Generalized lymphadenopathy
IV	Liver and/or spleen involvement with or without stage I to III
V	Involvement of BM, kidney, CNS, eyes, heart with or without stage I to IV

Table 6. Samples assessed in the current study.

Normal lymphnodes	TRAP	TRAP ELISA	Histopathologic characteristic
1)	(-)	(-)	
2)	(-)	(-)	
3)	(-)	(-)	
4)	(-)	(-)	
5)	(-)	(-)	
6)	(-)	(-)	
7)	(-)	(-)	
8)	(-)	(-)	
9)	(-)	(+)	
10)	(-)	(-)	
11)	(+)	(-)	
12)	(-)	(-)	
13)	(-)	(+)	
Lymphomas			
1)	(+)	(+)	lymphoblastic
2)	(+)	(+)	
3)	(+)	(+)	lymphoblastic
4)	(+)	(+)	Intermediate grade
5)	(+)	(-)	
6)	(+)	(-)	lymphoblastic
7)	(+)	(+)	High grade
8)	(+)	(+)	Intermediate grade
9)	(+)	(+)	Diffuse large cell
10)	(-)	(-)	Low grade
11)	(+)	(+)	Large cell
12)	(-)	(+)	
13)	(+)	(+)	B-cell
14)	(+)	(+)	T-cell
15)	(-)	(-)	Suspicious of lymphoma
Reactive Nodes			
1)	(-)	(-)	
2)	(-)	(-)	

Table 7. Sensitivity and specificity results for the TRAP assay

		Histopathology		Total
		Lymphoma	Non-lymphoma	
		Positive	Negative	
TRAP	Positive	12 (a)	1 (b)	13 (a+b)
	Negative	3 (c)	14 (d)	17 (c+d)
Total		15 (a+c)	15 (b+d)	30 (a+b+c+d)

Table 8. Sensitivity and specificity for the TRAP ELISA results

		Histopathology		Total
		Lymphoma	Non-lymphoma	
		Positive	Negative	
TRAP ELISA	Positive	11 (a)	2 (b)	13 (a+b)
	Negative	4 (c)	13 (d)	17 (c+d)
Total		15 (a+c)	15 (b+d)	30 (a+b+c+d)

Table 9. Cohen agreement coefficient for the TRAP and TRAP ELISA test.
Observed agreement between the two tests = (a+d)/(a+b+c+d) = (10+14)/30 = 80%
Cohen Agreement Coefficient (K) = 0.59 95% CI: 0.30-0.88
This coefficient measures the agreement between two different classification systems unbound from the agreement obtained by chance. The highest agreement is achieved for K values = 1

		TRAP		Total
		Pos	Neg	
TRAP ELISA	Positive	10 (a)	3 (b)	13 (a+b)
	Negative	3 (c)	14 (d)	17 (c+d)
Total		13 (a+c)	17 (b+d)	30 (a+b+c+d)

Table 10 Monte Carlo Simulation: simulation of the probability to have a tumor after a positive result on TRAP ELISA with the following scenario: generalized lymphadenopathy, cytology positive sample, probability to have a tumor before the test = 80%, and sensitivity and specificity assessed with a β-distribution.

K	**Interpretation**
< 0	No Agreement
0.0 – 0.19	Poor Agreement
0.20 – 0.39	Fair Agreement
0.40 – 0.59	Moderate Agreement
0.60 – 0.79	Substantial Agreement
0.80 – 1.00	Almost Perfect Agreement

Table 11. Post-test probability outcome obtained with the Monte Carlo simulation method.

Parameters	Values	Formula
Sensitivity	0.71	RiskBeta(12; 5)
Specificity	0.82	RiskBeta(14; 3)
LR+	4	Se/ (1-SP)
Prob pretest (P)	0.80	Subjective prob
Odds pretest (OptT)	4	P/(1-P)
Odds posttest (OpsT)	16	OpsTxLR+
Prob posttest	0.94	OpsT/ (1+OpsT)

CHAPTER 8. FIGURES

Fig 1. TRAP Specificity

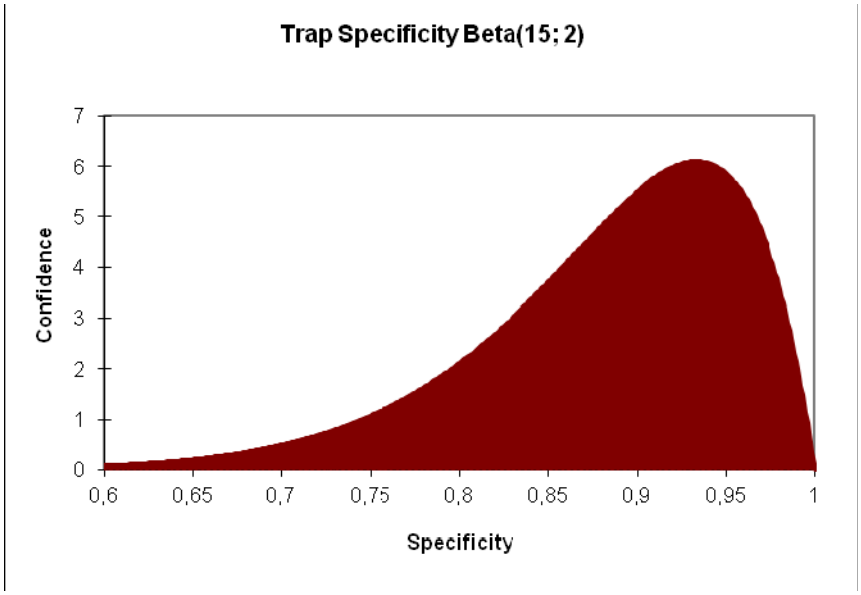


Fig 2. TRAP Sensitivity; Diagnostic Odds = (a*d)/(c*b) = 56

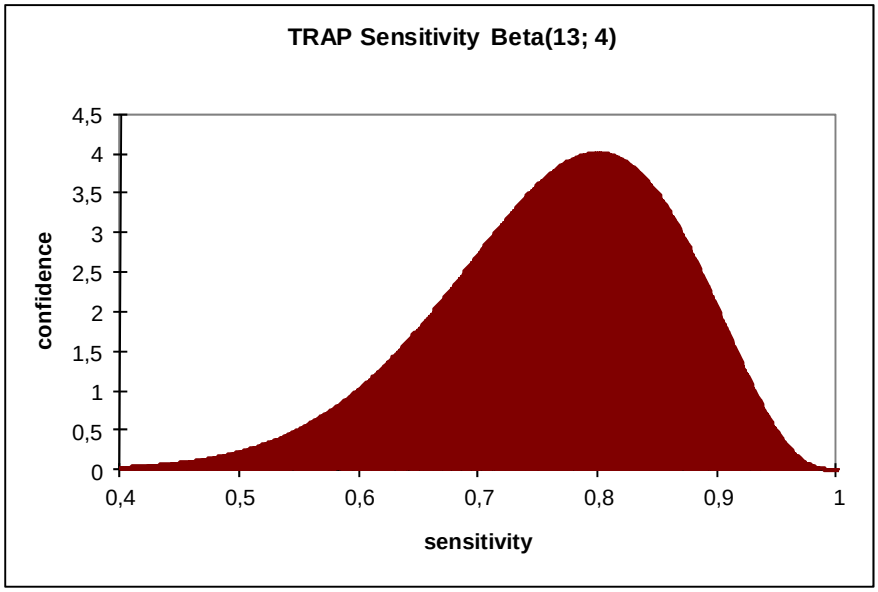


Fig 3. TRAP ELISA Sensitivity

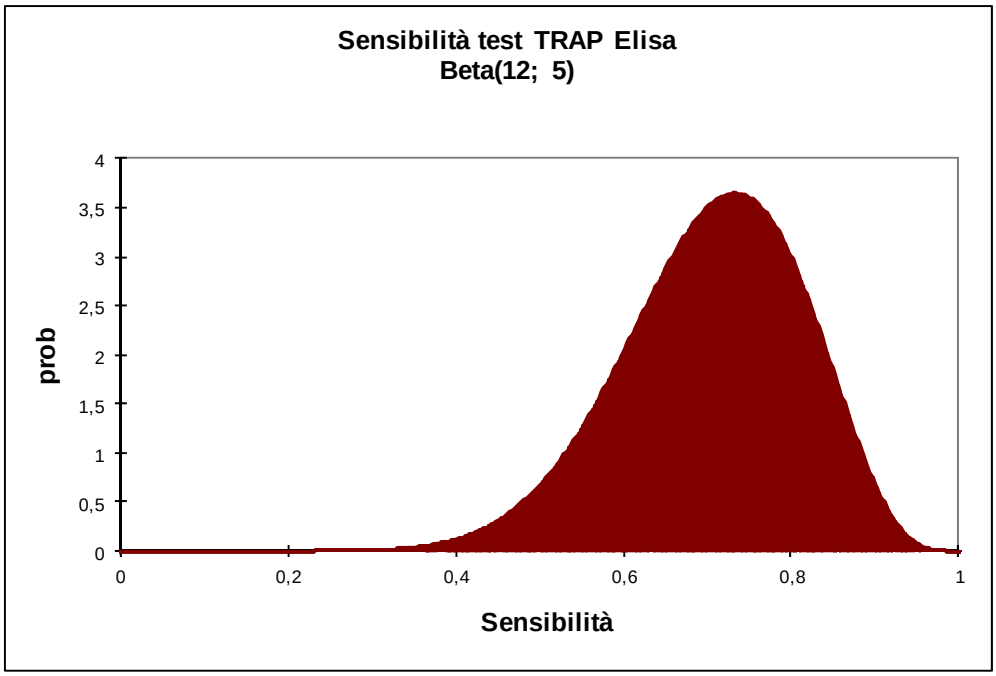


Fig 4. TRAP ELISA Specificity

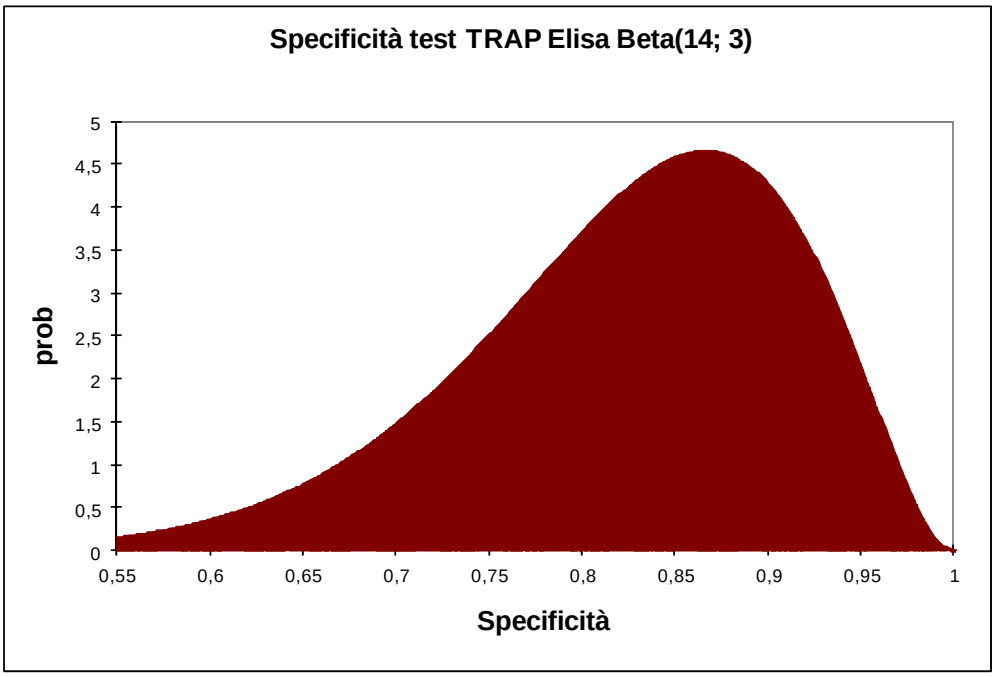
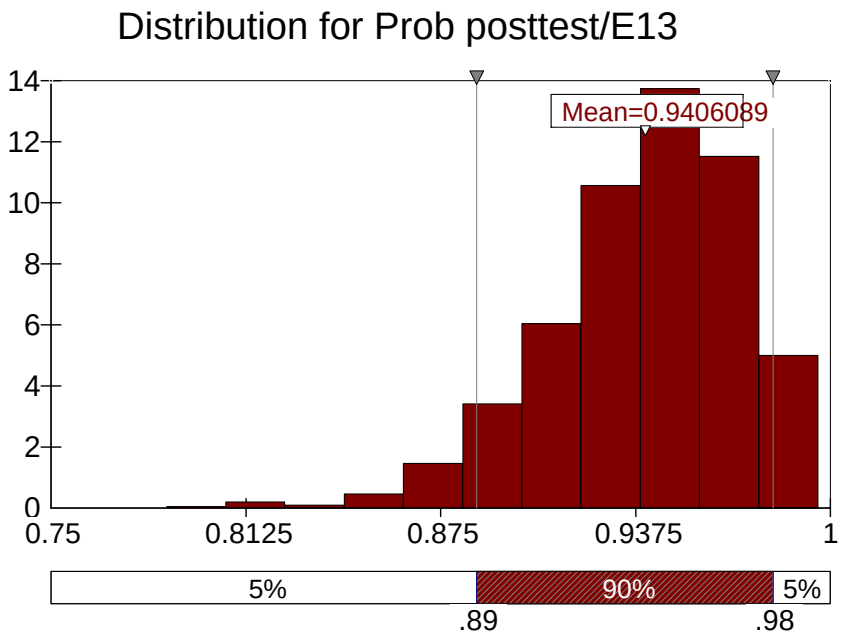


Figure 5. Distribution of the post-test probability.

Quartile distribution:

Min	25%	Median	75%	Max
79%	92%	94%	96%	99%



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