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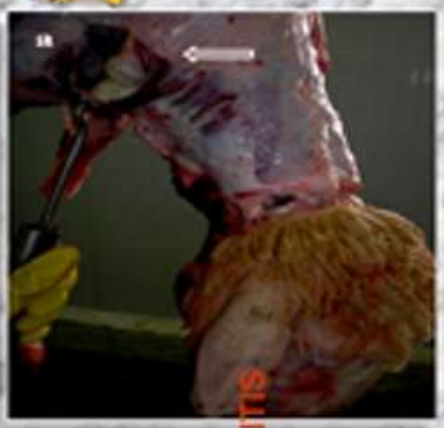

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Dear Esteemed Readers, Authors, and Colleagues,

I hope this letter finds you in good health and high spirits. It is my distinct pleasure to address you as the Editor-in-Chief of Integrative Omics and Applied Biotechnology (IIOAB) Journal, a multidisciplinary scientific journal that has always placed a profound emphasis on nurturing the involvement of young scientists and championing the significance of an interdisciplinary approach.

At Integrative Omics and Applied Biotechnology (IIOAB) Journal, we firmly believe in the transformative power of science and innovation, and we recognize that it is the vigor and enthusiasm of young minds that often drive the most groundbreaking discoveries. We actively encourage students, early-career researchers, and scientists to submit their work and engage in meaningful discourse within the pages of our journal. We take pride in providing a platform for these emerging researchers to share their novel ideas and findings with the broader scientific community.

In today's rapidly evolving scientific landscape, it is increasingly evident that the challenges we face require a collaborative and interdisciplinary approach. The most complex problems demand a diverse set of perspectives and expertise. Integrative Omics and Applied Biotechnology (IIOAB) Journal has consistently promoted and celebrated this multidisciplinary ethos. We believe that by crossing traditional disciplinary boundaries, we can unlock new avenues for discovery, innovation, and progress. This philosophy has been at the heart of our journal's mission, and we remain dedicated to publishing research that exemplifies the power of interdisciplinary collaboration.

Our journal continues to serve as a hub for knowledge exchange, providing a platform for researchers from various fields to come together and share their insights, experiences, and research outcomes. The collaborative spirit within our community is truly inspiring, and I am immensely proud of the role that IIOAB journal plays in fostering such partnerships.

As we move forward, I encourage each and every one of you to continue supporting our mission. Whether you are a seasoned researcher, a young scientist embarking on your career, or a reader with a thirst for knowledge, your involvement in our journal is invaluable. By working together and embracing interdisciplinary perspectives, we can address the most pressing challenges facing humanity, from climate change and public health to technological advancements and social issues.

I would like to extend my gratitude to our authors, reviewers, editorial board members, and readers for their unwavering support. Your dedication is what makes IIOAB Journal the thriving scientific community it is today. Together, we will continue to explore the frontiers of knowledge and pioneer new approaches to solving the world's most complex problems.

Thank you for being a part of our journey, and for your commitment to advancing science through the pages of IIOAB Journal.

Yours sincerely,

Vasco Azevedo

Vasco Azevedo, Editor-in-Chief
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COMMENTARY: NEUROSCIENCE AND GENETICS

UNDERSTANDING THE HIGH MIND: HUMANS ARE STILL EVOLVING GENETICALLY

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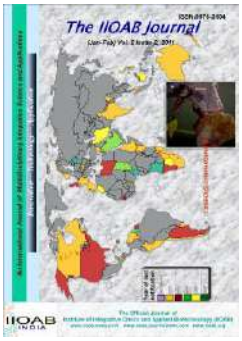
ABSTRACT



The total population of the United States at the turn of the 21 st century was 281,421,906. The total number of people above the age of 12 years old was estimated at 249 million. The National Institutes on Drug Abuse and the Substance Abuse and Mental Health Services Administration (SAMHSA) have surveyed persons age 12 and older and found that in the year 2001, a total of 104 million people have used illegal drugs in their life (ever used), 32 million used a psychoactive drug in the past year (2000-2001) and 18 million used a psychoactive drug in the past 30 days. Interestingly this does not include Alcohol. We must ask then, who are the people that could just say NO? When almost half-of the US population have indulged in illegal drug practices, when our presidential candidates are forced to dodge the tricky question of their past history involving illegal drug use, and when almost every American has sloshed down a martini or two in their life time, there must be a reason, there must be a need, there must be a natural response for humans to imbibe at such high rates. There is even a more compelling question surrounding the millions who seek out high risk novelty. Why do millions have this innate drive in face of putting themselves in harms-way? Why are millions paying the price of their indiscretions in our jails, in hospitals, in wheel chairs and are lying dead in our cemeteries. What price must we pay for pleasure seeking or just plain getting "HIGH"? Maybe the answer lies within our brain. Maybe it is in our genome? Utilization of the candidate vs the common variant approach may be parsimonious as it relates to unraveling the addiction riddle. In this commentary we have discussed evidence, theories and conjecture about the "High Mind" and its relationship to evolutionary genetics and drug seeking behavior as impacted by genetic polymorphisms. We consider the meaning of recent findings in genetic research including an exploration of the candidate vs the common variant approach to addiction, epigenetics, genetic memory and the genotype-phenotype problem. We speculate about the neurological basis of pleasure seeking and addiction, the human condition and

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the scope of societal judgments that effect multitudes in a global atmosphere where people are seeking "pleasure states".

Keywords: *evolution; genome; common variant hypothesis; candidate genes; reward deficiency syndrome (RDS); dopamine*

The mind of man is capable of anything- because everything is in it, all the past as well as all the future.

Joseph Conrad
Heart of Darkness

[I] INTRODUCTION

A fundamental premise about the brain is that its workings – sometimes referred to as the "mind" – are the result of its anatomy, physiology, neurochemistry, and genome, and that is all. The mind, therefore, is a consequence of the brain, and of the action of its constituents. Some people have difficulty accepting that premise, whether as an outgrowth of their religious upbringing, or their acceptance of other premises springing from tradition or folklore, their belief is that the mind is something magical. They perceive it as unidentifiable and quite separate from the body, as if it were made of quite different "stuff". Some even refer to it as the "soul." [1]

It is not our intent to argue or defend the mind/body dualism theories of the early days of psychology. Rather, this is an attempt to understand the mind in terms of alterations in its' level of function.

It is interesting that as early ***Homo sapiens*** spread out from Africa starting around 60,000 years ago, they encountered environmental elements and challenges that they could not overcome with prehistoric technology. In this regard many scientists falsely expected that analyses of our genome would indicate that an explosion of evolutionary mutations would have spread quickly throughout different populations. They theorized that these beneficial mutations would confer a greater survivability. However recent analysis of by Jonathan K Pritchard [2] and others [3] suggest that recent human evolution has occurred at a far slower pace than biologists had envisioned. While there have been examples of strong genetic mutations that have resulted in adaption to environmental pressure such as observed in Tibet. There transition to high altitudes resulted in gene mutation that favors this environmental shift in Tibetans [4]. The genome actually contains few examples of very strong rapid natural selection. Instead most of the visible natural selection appears to have occurred over tens of thousands of years. It is noteworthy that the rate of change of most traits is very slow indeed and as such major adaptive shifts require stable conditions across millennia. According to Pritchard [5]: "***Thus 5,000 years from now the human milieu will no doubt be very different. But in the absence of large-scale genomic engineering, people themselves will probably be largely the same. With this in mind then it may not be surprising to find a fairly high genetic influence on drug/pleasure seeking behavior in Homo sapiens possibly due to carrying a gene form that sets–up the individual to be driven by a need to up-regulate specific proteins such as dopamine receptors to induce a feeling of well-being and happiness".***

[II] THE GENE MANUAL

Most recently we have seen an enormous advance in the breaking of the genetic code and the identification of some 30,000 genes in the human genome. The gene manual is now known to contain a parts list of about 20,000 genes –strings of DNA letters that spell out the information required to build proteins. About 2 percent of the human genome encodes proteins, and a roughly similar amount seems to be involved in gene regulation. Most of the rest of the genome has as yet no known role. Modern research suggests that the entire scope of recent history and biology points to an inescapable conclusion**:** that we are, to a remarkable degree, chemically identified (made-up) of nucleic acids, DNA and RNA, and other operational agents referred to as proteins as either receptors or enzymes [5,6].

The basic needs of humans include sustenance, reproduction and safety .These needs are challenged on a daily basis and for many people replaced with hunger, lack of human contact and fear. Many drugs that have abuse potential and other natural stimuli such as food or sexual activity produce similar chemical changes in the brain. That change is an increase in extracellular dopamine (DA) in the shell of the nucleus accumbens (NAc). Moreover, the reward mechanism is at least very similar for all stimuli.

Presently the available information shows that the reward pathways involved are complex and have multiple elements. Multiple brain regions, multiple receptors, multiple distinct neurons, multiple transmitters, multiple transporters circuits peptides, proteins, metabolism of transmitters, and phosphorylation, all participate in reward mechanisms. Most

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recently, a review of published literature on fMRI studies of love, illustrating brain regions associated with different forms of love revealed interesting results. Although all fMRI studies of love point to the subcortical dopaminergic reward-related brain systems (involving dopamine and oxytocin receptors) for motivating individuals in pair-bonding, a meta analysis newly demonstrated that different types of love involve distinct cerebral networks, including those for higher cognitive functions such as social cognition and bodily self-representation [7].

The sets of mechanisms involved in the reward from different drugs of abuse, are different from the mechanisms in the reward from natural stimuli such as food or sexual activity; thus there are different systems that distinguish different stimuli. Separate functions of the reward system such as anticipation, evaluation, consummation and identification; all contain function-specific elements. The level of the stimulus also influences the participation of the elements in the reward system. There are possible reactions to even below threshold stimuli, and excessive stimuli can change reward to aversion. Learning and memory of past reward is an important and integral element involved in reward and addictive behavior. Many of the reward elements are altered by repeated or chronic stimuli, and chronic exposure to one drug is likely to alter the response to another stimulus. To evaluate and identify the reward stimulus thus requires understanding of the heterogeneity of the reward components in the brain [8].

[III] HUMAN GENOME PROJECT: REVOLUTION OR FAILURE?

If the conclusion that we are made up basically of genetic material is feasible, and as such we are very complex then what makes us so different from other organisms of the universe? Is it our "mind" or is it the evolutionary impact of the environment on our genetic material . Do these concepts meld into a hybrid blend of biological and functional activity? We do now understand that there are mutations in various genes which have occurred ancestrally and are referred to as older gene forms, and there are other mutations in the same gene that are referred to as new gene forms. We also know that certain environmental elements could actually effect the expression of certain gene forms whether old or new. For example, Vitamin B12 can add methyl groups on a gene and change its expression. There are also epigenetic effects influencing drug seeking behavior, ADHD and even schizophrenia [9] [see epigenetics section below].

The Human Genome Project opened the pathway to solving the mysteries of disease, after a decade of arduous research from world class scientists while the future looks bright, there is a long way to go before we could achieve medical "miracles".
To many the Human Genome Project has failed so far to provide the medical miracles that scientists promised in 2000. In that year leaders of the Human Genome Project announced completion of the first rough draft of the human genome. One of the predictions was that follow-up research could pave the way to personalized medicine within ten years. Accordingly to date few medical applications have emerged but important insights have already revolutionized medical research including psychiatric genetics. While some leading geneticists argue that a key strategy known as *the "common variant" hypothesis* for seeking medical insights into complex diseases such as addiction is fundamentally flawed, others say that the strategy is valid [10].

The obvious way to see who was right would have been to sequence the full genome of diseased and healthy individuals and, using powerful computers, identify DNA variations that turned up in patients with the given disease but not in control subjects. Using sophisticated techniques many scientists embarked on large scale studies, known as *genome-wide association studies* (often called GWAS) that relied on landmarks in DNA known as single-nucleotide polymorphisms or SNPS, to uncover gene variants important in disease.

These concepts led to the development of HapMap. [11]. Thus in the last five years genome-wide association studies have looked at hundreds of thousands of common SNPS in the genome of tens of thousands of individual subjects and controls in search of SNPS linked to common disease. While a number of leaders in the field believe that using this strategy has revealed important clues and uncovered pathways for a number of common diseases such as schizophrenia, type 2 diabetes, Alzheimers and hypertension no magic bullets have been developed. In fact it is astounding that being able to crack the human Genome allowing scientists to look at the entire compliment of common genetic variants has not led to any major breakthroughs especially in neurobiology. Moreover, in a recent interview [12] David Botstein of Princeton University (Hall 2010) discussing the HAPMAP stated "It had to have (been) done. If it had not been tried, no one would have known that it didn't work" The 138 million HapMap he says was a "magnificent failure". This has been further supported by Walter Bodmer who first proposed the genome project. He also believes that the common variant hypothesis is a dead end and suggests that the vast majority of common variants have shed no light on the biology of disease [12].

The current argument over the common variant hypothesis suggests at least one way forward for solving what many are calling the "missing" heritability problem. That is to search for rare genetic variants.

However, it is plausible that new approaches should consider re-exploring the older candidate approach instead of relying on GWAS for an answer. In this approach scientists pick and choose genes to examine in those people, based on prior knowledge of physiological processes as Blum and Noble [13] did in search of the "reward gene." Using this approach they found at least one important variant of the dopamine- D2

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receptor gene (Taq ***A1***) that is associated with severe alcoholism. This apparently simple finding is complicated by the fact that many other genes in specific brain reward pathways [14] and their polymorphisms all work in concert to provide the end – genetically controlled phenotype. In this regard, Li [15] proposed that 396 genes work in a network of common pathways to influence the final net release of Dopamine and Glutamate in the reward center thus effecting drug seeking behavior.

3.1. Epigenetic changes

Interestingly, Joseph Nadeau, director of scientific development at the Institute for Systems Biology in Seattle, has tracked more than one hundred biochemical, physiological and behavioral traits that are affected by epigenetic changes. In addition, he has seen some of these changes passed down through four generations. This phenomena, which has been called the *"genotype-phenotype problem"* evokes the potential importance of the environment. However we have no evidence that in terms of addiction liability, for example, of what impact elements in society play in and to what extent these epigenetic effects impact future generations. However, we must keep an open mind and also realize that transgenerational genetic effects impact phenotypic expression and may confer disease risk [16]. Nadeau et al [16] suggests that some common illnesses may ultimately be traceable to a very large number of genes in a network or pathway whose effects may each vary. Various effects may depend on the gene variant whereby the presence of one gene variant can exacerbate or counteract the effect of another disease –related gene in the group. This takes on even more importance especially for the addiction field when we evoke the so called "domino effect" as observed in the cascade associated with brain reward" [14]. While we have not as yet seen medical miracles the genome project opened the doors and we should not predict a timetable for such miracles to occur but just imagine what Darwin and Mendel, for example, could do with this technology.

[IV] DNA AND REINCARNATION: GENE-ENVIRONMENTAL INTERACTION

In his book on the evolution of human intelligence, "The Dragons of Eden, " the late Carl Sagan as early as 1977, speculated that most complex organisms on earth today contain substantially more stored information, both genetic and extra-genetic, than the most complex organisms of two hundred million years ago. According to Sagan, the basic means for capturing this information lies within something termed "genetic memory" [17].

All organisms on planet Earth have chromosomes which contain the genetic material passed on from generation to generation, whether those organisms are fruit flies or human beings [18]. Indeed, there are those who speculate on the process of reincarnation as being nothing more than the passage of information from one life form to another life form via DNA molecules. While no specific gene polymorphisms have ever been associated with an affinity for reincarnation per se, recent serious research on the relationship between child abuse and reincarnation has been investigated [19].

To gain an understanding of the psychosocial function of reincarnation among Druze, interviews were conducted with nine male subjects who had experienced reincarnation and with one or two of their family members. Analysis of these interviews revealed that the onset of experienced reincarnation typically occurs at between two and five years of age. Five of the subjects had displayed psychological distress in their childhood that was alleviated after the experience reincarnation.

Once the child has displayed initial indications of reincarnation, such as mentioning names that the family construes as being from a past life, the family takes an active role in constructing the past-life story and matching it to a known real story involving a tragic death. This match creates a new order in the life of the child, the family, and the past-life family. The findings support the sociocognitive notion of the constructing of past memories by the social environment.

In subsequent sections we discuss the relationship of genetic polymorphisms and potential positive and negative impacts on reward mechanisms that could influence long-term survival. An important question to ponder is why there is an increase in aggressive, addictive and other behavioral disorders and is our species in peril? In the past these trends have been attributed purely to environmental factors and to the stress of our increasingly complex and technological society. A new theory is emerging that just the opposite is occurring that our increasingly complex society, with its requirement for more and more years of higher education to compete, is selecting for the genes associated with these "reward" behavioral disorders, and that these genes are increasing infrequency in our society. In essence a breakdown of brain reward functioning has board implications for public policy –as well as the future of the human species.

It is feasible that a cave man imbibing his ***mandragova*** officianarum (mandrake root), a psychoactive substance with extreme aphrodisiacal powers, may have experienced an effect which was passed through "genetic memory" to his offspring and to subsequent offspring. The experience, being pleasant and stored, may or may not be experienced in the recipient offspring. However, appropriate extra-genetic stimuli may trigger awareness of that stored pleasure state, in one degree or another, for future generations.

Given that extra genetic triggering action (could be certain chemicals, toxins, etc); the recipient offspring may believe it to be a "fantasy" or "hallucinations", whereas in reality the experience may have its origin as far back as recorded history, or as far back as the initial ingestion of the mandrake root.

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[V] INSIDE THE BRAIN

"Of all animals, man has the largest brain in proportion to his size"

Aristotle
The Parts of Animals

With evolution came genetic mutations, and with mutations came ***Homo sapiens***. Where is this genetic information stored? To protect against accident, we would expect natural selection to have evolved substantial redundancy, it is also scientifically apparent that the brain is not equipotent in its capacity to store bits of information for memory purposes.

Studies on the limbic system reveal that electrical activity of that part of the brain, termed "theta activity" appears to be related to short-term memory. Drugs which increase Theta Activity also have been found to increase memory [20].

A review of pertinent literature makes it difficult to resist the conclusion that – at least in humans –memories are stored somewhere in the cerebral cortex and activated within the limbic system. They involve chemical messengers, dopamine, gallanin, acetycholine as well as the *fos* gene and wait to be retrieved by electrical impulses generated through endogenous substances or processes within the brain itself or the ingestion of psychoactive substances including drugs of abuse.

An increasing body of evidence shows that structural modifications of chromatin, the DNA-protein complex that packages genomic DNA, do not only participate in maintaining cellular memory (e.g., cell fate), but they may also underlie the strengthening and maintenance of the synaptic connections required for long-term changes in behavior. Accordingly, epigenetics has become a central topic in several neurobiological fields such as memory, drug addiction, and several psychiatric and mental disorders [21].

This interest is justified as dynamic chromatin modifications may provide not only transient but also stable (or even potentially permanent) epigenetic marks to facilitate, maintain, or block transcriptional processes, which in turn may participate in the molecular neural adaptations underlying behavioral changes. Through epigenetic mechanisms the genome may be indexed in response to environmental signals, resulting in specific neural modifications that largely determine the future behavior of an organism. In their review Malvaez et al. [21] discuss recent advances in our understanding of how epigenetic mechanisms contribute to the formation of long-term memory and drug-seeking behavior and potentially how to apply that knowledge to the extinction of memory and drug-seeking behavior.

[VI] THE HEMISPHERE CONNECTION

The human brain consists of a right and left hemisphere, both of which are connected via the corpus callosum. The complex cabling system represented by the corpus callosum underlines the fact that although the separate hemispheres have separate functions the interaction of the hemispheres is a vital human function which may have impact on one's behavior such as, for example obsessive compulsive disorder [22].

Humans exhibit an interesting separation of musical and verbal skills [23]. Patients with lesions of the right hemisphere are significantly impaired in musical, but not verbal, ability. However, even when performance of musical abilities is hampered by right hemisphere lesions, the ability to read music is unimpaired.

In this regard, it has been stated by Sagan that evidence from scientifically sound experiments suggest that those functions we ordinarily describe as "rational" live mainly in the left hemisphere, while those we consider "intuitive" dwell mainly in the right [17].

As early as the 70's, Robert Ornstein and David Galin of the Lanley Porter Neuropsychiatric Institute of San Francisco claim that as normal people change from analytic to creative intellectual activities, the EEG activity of the corresponding cerebral hemispheres varies in the predicted way: for example,when a subject is performing mental arithmetic, the right hemisphere exhibits the alpha rhythm characteristic of an "idling" cerebral hemisphere [24].

According to Ornstein and Galin, the Western world is left hemisphere oriented. They suggests that our awareness of right hemisphere function is a little like our ability to see stars in the daytime. Sagan further describes Ornstein's hypothesis:

The sun is so bright that the stars are invisible, despite the fact that they are just as present in our sky in the daytime as at night. When the sun sets, we are able to perceive the stars. In the same way, the brilliance of our most recent evolutionary accretion, the verbal abilities of the left hemisphere, obscures our awareness of the functions of the intuitive right hemisphere, which in our ancestors must have been the principle means of perceiving the world [17].

In essence, then, inhibition of the left hemisphere releases our potent "intuitive" side of the brain, reflecting our most primitive desires, feelings and appetites. This has been described by some as the coming out party of our '*reptilian brain*"

In 1962, William Barrett, in his *Irrational Man,* described the historical transition from intuitive to rational dominance of human consciousness as a traumatic turning point in human development, both as a source of power on one hand, and as an alienation from more primitive powers on the other.

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"This capacity for loving easily and familiarly at an extraordinary level of abstraction is the source of modern man's powers. With it he has transformed the planet, annihilated space, and trebled the world's population. But it is also a power which has, like everything human, its negative side, in the desolating sense of rootlessness, vacuity, and lack of concrete feeling that assails modern man in his moments of real anxiety" [26].

It is not surprising that such moments of real anxiety should drive modern man to experimentation with consciousness-altering substances with hopes that can touch the roots of his intuitive powers.

For example, since the early sixties and now even in the first part of the millennium, a closer look at the plant psychotropic marijuana reveals that it is often described as improving our appreciation for music, the arts, dance, sex, sign and symbol recognition and our sensitivity for nonverbal communications. Today we know that cannabinoid receptors in the brain are laid down by certain genes .The brain cannabinoid receptor (CB1) was first isolated in 1988 by W.A. Devane and associates. Then in 1990, L.A. Matsuda, cloned the CB1 receptor gene (CNR1). Some have thought that short–term memory is lost with high doses of cannabinoids. This assertion has been borne out because there is a relationship between a decrease amplitude of a certain evoked related potential (ERP) called the P300 and frequent marijuana smokers. Decreased amplitude of the P300 and prolonged latency has already been associated with memory deficits as well as alcohol and drug dependence. Nevertheless, this effect of marijuana has not kept millions from its daily use. There are even some who proclaim that they function better while using it. For example, some have called for individualized substance use counseling for persons suffering from schizophrenia, citing the observation that in some cases use of marijuana might help a client "feel calmer and happier" and actually prevent stress-related relapse.[25] For yet other patients, the drug might have deleterious effects. Advocating this degree of individualization of treatment takes considerable courage and is certainly not the conventional wisdom or the textbook approach.

In this regard, however, marijuana is seldom, if ever, reported to improve our cognitive abilities. It seems a certainty that marijuana does not enhance our abilities for cognitive calculation, whether represented by our appreciation of Einstein's theory of relativity, the calculation of a rocket trajectory, or the computation of a Nernst equation [26].

It is more commonly observed that rather than enhancing human powers, the cannabinols (active ingredients of marijuana) simply suppress the left hemisphere and permit the stars to come out. Such inhibition to the left hemisphere may be similar for other psychoactive substances, such as opiates derived from poppy, cocaine from cocoa root, alcohol from fermented sugar, nicotine from tobacco and sugar from sugar cane. The neurochemical similarity of all these left – hemisphere suppressors induce presynaptic dopamine release from the reward site of the brain in the *mesa-limbic system* called the *nucleus accumbens.*

This inhibition/release mechanism may even be the objective of not only the meditative states of many eastern religions it may also provide the basis for the seeking of pleasure states via natural or unnatural means. In other words, is the "high" we seek simply the suppression of the left hemisphere and preferential dopamine release?

Barrett has suggested that man's preoccupation with left-hemisphere concerns in contemporary society drives him to alienation and loneliness that must somehow be relieved.

"----man's feeling of loneliness or alienation has been intensified in the midst of a bureaucratized, impersonal mass society He has come to feel himself an outsider even within his own human society--- But the worst and final form of alienation--- is man's alienation from his own self. In a society that requires of man only (that) he performs competently his own particular social function, man becomes identified with this function, and the rest of his being is allowed to subsist as best it can- usually to be dropped below the surface of consciousness and forgotten"[26].

Likewise, Harvard neuroanatomist Jill Bolte Taylor described her subjective experience of the aftermath of her left-sided stroke in the following manner:

"Because I could not identify the position of my body in space, I felt enormous and expansive, like a genie just liberated from her bottle. And my spirit soared free like a great whale gliding through the sea of silent euphoria. I remember thinking there's no way I would ever be able to squeeze the enormousness of myself back inside this tiny little body. But I realized "But I'm still alive! I'm still alive and I have found Nirvana. And if I have found Nirvana and I'm still alive, then everyone who is alive can find Nirvana." I picture a world filled with beautiful, peaceful, compassionate, loving people who knew that they could come to this space at any time. And that they could purposely choose to step to the right of their left hemispheres and find this peace" [27]

The ways and means of returning to delve again into those forgotten regions of the subconscious which house "the rest of his/her being," are many and varied. Whether the "high" is sought to suppress left hemisphere dominance is "narcotic" or "natural", the end is identical. In a recent study by Baloch et al. [28] fifty one children and adolescents with DSM-IV Pediatric bipolar disorder and 41 healthy comparison subjects underwent 1.5 T structural magnetic resonance imaging brain scans. Exploratory analysis showed pediatric bipolar disorder subjects who had one or more first degree relatives with mood disorders had significantly smaller left hemisphere SGPFC compared to healthy controls ($p = 0.03$ Sidak corrected).

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From 1987 – 1990, one if us (KB) had the fortunate experience of directing a research project at the University of Texas Health Science Center in San Antonio, Texas, and UCLA in search for genes that may associate with severe alcoholism. In 1990, Ernest Noble (former Director of NIAAA) and KB published their findings in the prestigious Journal of the American Medical Association. In essence they found the first "defective" gene that associated with severe alcoholism. The press wrongly labeled the finding as "EXPERTS FIND THE ALCOHOLISM GENE" [13].

This was wrong because there is nothing in the brain that is particular to alcohol per se. Instead Blum and Noble and associates found a gene form that really associates with brain reward or feelings of well–being. After many years of additional research and a lot of controversy their work has now withstood the test of time. While there are many genes involved in causing impulsive, compulsive and addictive behavior, it is interesting that these genes work in concert and predispose an individual to these destructive behaviors. There is a common genetic thread that causes alcoholism, drug dependence, nicotine dependence, carbohydrate cravings, and other behaviors such as pathological gambling, sex addiction and even serial killing. Being perplexed with the field of biological psychiatry KB coined the term "Reward Deficiency Syndrome (RDS)" to describe this common genetic thread linking all the addictions [29].

So basically, the junkie on the street making a heroin run is neurochemically similar to the executive gulping down five martinis at a business lunch. The main difference is the stigma attached to the heroin addict compared to the respected business person. The reason why these individuals are neurochemically similar is that both heroin and alcohol stimulate the same brain reward site and cause the nerve cell release of a substance called dopamine. Regulation of dopamine plays a crucial role in our mental and physical health. In addition to affecting brain processes that control movement, emotional response, and ability to experience pleasure and pain, Dopamine's two major functions are as an anti-stress molecule and a pleasure inducing molecule [30]

Drug addiction is a chronically relapsing disorder that has been characterized by (1) compulsion to seek and take the drug, (2) loss of control in limiting intake, and (3) emergence of a negative emotional state (for example, dysphoria, anxiety, irritability) reflecting a withdrawal syndrome when access to the drug is prevented. Drug addiction has been conceptualized as a disorder that involves elements of both impulsivity and compulsivity that yield a composite addiction cycle composed of three stages: 'binge/ intoxication', 'withdrawal/ negative affect', and 'preoccupation/ anticipation' (craving) [31].

Animal and human imaging studies have revealed discrete circuits that mediate the three stages of the addiction cycle. The ventral tegmental area and ventral striatum is a focal point for the binge/intoxication stage. The extended amygdale is the key area in the withdrawal/negative affect stage. The preoccupation/anticipation stage involves a widely distributed network including the orbitofrontal cortex-dorsal striatum, prefrontal cortex, basolateral amygdala, hippocampus, and insula, involved in craving and the cingulate gyrus, dorsolateral prefrontal, and inferior frontal cortices involved in disrupted inhibitory control [32].

The transition to addiction involves neuroplasticity in all of these structures that may begin with changes in the mesolimbic dopamine system and a cascade of neuroadaptations from the ventral striatum to dorsal striatum and orbitofrontal cortex and eventually dysregulation of the prefrontal cortex, cingulate gyrus, and extended amygdala. The delineation of the neurocircuitry of the evolving stages of the addiction syndrome forms a heuristic basis for the search for the molecular, genetic, and neuropharmacological neuroadaptations that are key to vulnerability for developing and maintaining addiction [31].

Thus, imagine the world without dopamine and you would find an individual caught up in a very anxious non pleasurable and totally frustrated state. When there is a lack of dopamine receptors the individual seeks out substances that will fix the problem by stimulating dopaminergic pathways.

[VII] BRAIN REWARD MECHANISMS

In his book "Alcohol and the Addictive Brain" with James Payne [33] KB provided a look at how the brain may function leading to either well-being/euphoria or dys-ease/dysphoria. Many more recent experiments confirm these ideas.

It is well known that in the brain reward site, the chemical messenger dopamine works to maintain our normal drives: hunger, thirst, and sex. In fact, dopamine has come to be known as the "pleasure molecule" and/or "anti-stress molecule." When dopamine is released into the synapse, it stimulates a number of dopamine receptors (D1-D5) that results in a feeling of well-being and stress reduction. This is the result of the interaction of numerous transmitters-serotonin (5HT), endorphins (END), GABA (GB), dopamine (DA), norepinephrine (NE), and acetycholine (ACH).

The process of the interactions at the brain "reward site" is called: the reward cascade [14]. A consensus of the literature suggests that when there is a dysfunction in the "brain reward cascade," especially in the dopamine system causing a low or hypo-dopaminergic trait, the brain of that person requires dopamine to feel good. This high-risk genetic trait leads to multiple drug-seeking behaviors. This is so because alcohol, cocaine, heroin, marijuana, nicotine and glucose all activate release of dopamine, which can heal the abnormal cravings [34]. Moreover, this genetic trait is due to a form of a gene (DRD2A1 allele), which prevents the expression of, the normal laying down, of dopamine receptors in the brain reward site [13].

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This gene and others involved in neurophysiological processing reward neurotransmitters (i.e. 5HT, END, GB, DA, NE, ACH etc), have been associated with deficient functions and predispose individuals to have a high risk for addictive, impulsive, and compulsive behavioral propensities. These include: severe alcoholism, cocaine, heroin, marijuana, and nicotine addictions, glucose bingeing, pathological gambling, sex addiction, ADHD, Tourette syndrome, autism, chronic violence, post traumatic stress disorder, schizoid avoidance disorder, conduct disorder, and antisocial behavior [35].

It has been proposed that genetic variants of the D2 dopamine receptor gene and other "reward genes" are important common genetic determinants of the emerging concept Reward Deficiency Syndrome (RDS) [36]. Ongoing current research involved in chromosomal marking and candidate gene analysis has supported the concept of "polygenic inheritance" and epistasis. While certain pharmaceutical approaches include targeting of single neurotransmitter deficits (e.g. SSRI's), as well as blocking dopaminergic activity to reduce drug effects (e.g. Haloperidol), our approach includes multiple neuropharmacological targets and enhancement of dopaminergic function as a life-long goal.

Gene therapy studies by Nora Volkow revealed that over expression of D2 receptors in the NAc of alcohol drinking rodents results in a significant reduction of both alcohol preference and craving [37]. Similar findings have been obtained in animals with high cocaine preference [38]. While the goal of treatment is the early diagnosis of one's genetic propensity to substance seeking behavior with the possible potential of CNS gene therapy, current diagnosis and treatment includes, limited non-invasive DNA testing as well as precursor amino-acid -enkephalinase inhibitory therapy [39,40].

We propose that, based on this previous evidence, substance abuse treatment must involve physiological, psychological, and spiritual modalities. With reference to the physiology, we propose a biogenetic model for the diagnosis, treatment and prevention of relapse for RDS behaviors. Thus, genotyping, pharmaceutical interventions, nutraceutical therapies, neurofeedback, auricular therapy, acupuncture, chiropractic and certain natural forms of healing provide a unifying approach to reduce aberrant cravings and enhance recovery and well being by altering brain chemistry.

While the above is based on some scientific concepts, there are other approaches that can be used to look at the age old question of "why do we like to get "HIGH"? Before we suggest a further answer to the question of the why and how of "High", there are other concepts which must first be taken into considerations.

[VIII] THE TRIUNE CONCEPT

Historically, MacLean in 1973, pointed out three basic components of the human brain, which for the most part have not really changed over the last 30 years, except for more defined functions and the discovery of a plethora of exciting genomic/phenotype topology. These functions include: 1) neocortex, 2) limbic and 3) reptilian complex (R-Complex). [41].

Accordingly it has been shown that the R-Complex plays an important role in aggressive behavior, territoriality, ritual and the establishment of social hierarchies. In some sense, the R-complex still performs dinosaur functions.

The limbic system generates strong or vivid emotions. Electrical discharges in the limbic system result in signs similar to those of psychoses or those produced by psychedelic or hallucinogenic drugs. The limbic system is the site of reward behaviors. It is the site where our desire for pleasure takes place. In a sense it is the pleasure center and by the exaggerated release of neuronal dopamine or possibly even opioid release, we perceive euphoria. All cravings occur at this site.

Human action is strongly influenced by expectations of pleasure. Making decisions, ranging from which products to buy to which job offer to accept, requires an estimation of how good (or bad) the likely outcomes will make us feel. Yet, little is known about the biological basis of subjective estimations of future hedonic reactions. Sharot et al [42] show that administration of a drug that enhances dopaminergic function (dihydroxy-L-phenylalanine; L-DOPA) during the imaginative construction of positive future life events subsequently enhances estimates of the hedonic pleasure to be derived from these same events. According to the authors these findings provide the first direct evidence for the role of dopamine in the modulation of subjective hedonic expectations in humans. The human cerebral neocortex is believed to be the seat of human cognition. It is frequently discussed in terms of four major regions or lobes: ***frontal, parietal, temporal and occipital lobes.*** Lesions in the neocortical areas often destroy initiative and creative traits.

Many Psychiatrists now believe that understanding the triune concept or brain part functions, and its interactions, albeit having separate but interrelated functions of the brain, may hold clues to the relationship between drugs and induced dreamlike states.

Based on the triune brain model, a new approach to psychopathology has taken shape. It is the evolutionary perspective of mental diseases such as the major psychoses, anorexia nervosa, anxiety disorders, and also brain diseases such as Parkinson's disease or Huntington's disease. Many mental illnesses are marked by severe deficits in social behavior and social communication. The social communication system disintegrates, especially in the major psychoses. The response choices to social or other external signals in a given situation

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become limited or even distorted, and reasoning is no longer part of decision making [43].

The emphasis of this contribution is on the disintegration of social behavior in psychopathology, based on evolutionary psychiatry. In terms of mind-body -soul, MacLean's concept provides valuable insight for understanding the biological roots of human social behavior and communication. It is time to uncover the ties between the natural and the social sciences [43, 44].

[IX] DREAMS

Dreams may be a way in which humans communicate with the right hemisphere. A major aspect of the dream state might be the unleashing of the R-complex processes that had been suppressed by the neocortex during the wakeful periods.

One of the most famous dreams known to have solved a difficult intellectual problem is that of the German chemist Friederich Kekule von Stradowitz in 1865. The dream allowed Kekule to determine the molecular nature of the benzene ring. Kekule was dozing when he had a dream of dancing atoms in linear arrangement, and the tail of a chain of atoms attached itself to the head and formed a rotating ring.

This is an example of pattern recognition and not an analytic activity, and as such is typical of almost all of the famous creative acts accomplished in the dream state. These manifestations are right hemisphere and not left hemisphere activities.

In trying to understand the "high" mind, we might consider the possibility that psychotropic substances induce in the subject dream-like states of a creative nature- sometimes referred to as hallucinations. However, holding that thought, we can also glean wisdom from individuals who imbibe because they believe and maybe rightly so, that their most creative moments have occurred under the influence of one or more psychoactive drugs. Should we limit our minds? Should we think inside the box? Should we have bounds? Some of the best music, art and scripts have been generated by pleasure seekers with great talent that is unleashed without bounds. Lenny Kravitz wrote-*"I have a pocket full of money and a pocket full of- keys without any bounds"*. Maybe we should be able to fly away and feel good at will – what a thought! In essence, our mental freedom is all that we come into this world with.

To continue, it may follow that if indeed dreams are the result of right hemisphere dominance and if there is an alteration of the level of consciousness in a positive way (feeling good – being high) then the "high" one obtains from drugs or herbs may be due to their effect of inhibiting the left hemisphere, as suggested earlier.

Sagan points out that in dreams "we are sometimes aware that a small portion of us is placidly watching; often in a corner of the dream, there is a kind of observer" [17]. In a nightmare, we may say to ourselves: "This is only a dream". Maybe this entire universe is a dream and someone else is dreaming this dream and we are just a part of it!! This idea becomes more vivid when we consider the movie ***"Inception"***.

According to Bruce Charlton, the Peak experiences in science could therefore be considered the result of a 'significance alarm' going off in the brain and their objective value depends on the specialized cognitive quality of that specific brain. So scientists may be correct to take peak experiences seriously. Perhaps the best approach is to regard the scientific peak experience as a signal from the self to the self (occurring in a dream state), a subjectively evaluated and auto-administered emotional reward for good thinking [45].

It is noteworthy that in psychodelic drug experiences –for example liquid Tetrahydrocannibinol (THC) or lysergic acid (LSD) the presence of such a "watcher" is frequently reported. Even today, years after Hoffman, Huxley, Leary, Ram Das, and Cohen, LSD experiences may be quite terrifying, and it is the "watcher" who acts to buffer the terrifying experiences. The "watcher" would say: "Hey man- this is just a dream". "The easy induction of pseudo-profound insights by intoxicants serves as a warning of the potential pitfalls. An arbitrary object becomes labeled with an obscure sense of delight and personal relevance in a process that could be termed the Colonel Flastratus! Phenomenon" [45]

As intelligent humans seeking knowledge attempting to uncover the mysteries of the mind, we must ask are drug-induced hallucinations fragmented bits of stored information having a direct link to our many pasts, or are they images of our future? Remember Conrad's statement –***"everything is in it (the mind), all the past as well as all the future-"***

[X] GETTING "HIGH"

In 1972, Andrew Weil, in the bestselling book ***"THE NATURAL MIND",*** [46] proclaimed that getting "high" is a time-honored tradition. Human beings are born with an innate need to get high, to experience periodically other states of consciousness, and the capacity for this experience is a capacity of the human nervous system. Often, external things, such as psycho active drugs, like strong marijuana, seem to cause highs, but this is an illusion**.** In fact it is due to the interaction of the drug and meso-limbic chemical messengers with the net effect of releasing dopamine at the reward site. Indeed it is a pleasurable activity likened to a pre-orgasmic experience increasing one's libido. Most importantly, the great thinkers and teaches of meditation, believe that it is possible to be high spontaneously and to learn to get high with less and less external stimulation.

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In terms of science the act of being high depends on one's receptor sensitivity to interaction with certain chemical messengers including serotonin, endorphins and dopamine. In reality unless there is sufficient quanta of the so called "high" molecules in the synapse, no high will be achieved. In essence the genome of each individual may have a lot to do with one's sensitivity, need and innate tolerance for the psycho active effects of any substance [47, 48].

Therefore, most people do not want to train their mind and slowly increase their ability to get high naturally. We must accept the fact that many people are going to rely on external things, including drugs and plants psychotropics, for their highs.

From the beginning, mankind has devoted considerable energy and ingenuity to "turning on" under such labels as "altering levels of consciousness" or as it use to be noted by Aldous Huxley the "opening the doors of perception". Through smoking, snorting, sniffing, eating, drinking, or main-lining, people of all cultures have sought a little more than the standard view of reality.

We could argue that today the problem of substance use disorder is symptomatic of more general societal problems, but we now know that certain gene forms can express a phenotype which actually predisposes an individual to wanting to get high compared to others that do not like to get high. In this regard, we know that individuals who carry the DRD2 A1 gene form love psychostimulants (cocaine) and those that carry the DRD2 A2 hate psychostimulants [49].

The continued understanding of neurogenetics and its role in drug seeking behavior will be the subject of many experiments in the future extending our existing knowledge of "Psychiatric Genetics".

[XI] DRUGS: HAVE IT YOUR WAY

The drug scene is nothing new and seems to be increasing in our younger generation. It is the American way, a trillion dollar complex that pushes and pumps its produce into all facets of our life. Burger King cannot compare to the blazing R_X sign in the sky. "If you have a pain, we have a pill", is not so odd to rejoin to, "Have it your way" (at Burger King'). The only real difference is that corporate drug dealers shy from the figures of how many "pill-burgers" they have actually dealt us. The question of "Should we give drugs to people?" -then takes on an expo factor value and echoes the empty ring of political "would have's, should have's". The real question at hand is, "What quantity of drugs can we afford" and what is the system of barter? Drugs or plant psychotropic abuse is not the real issue, people are. That people need other people is a basic human condition. Without interaction with another human, a person may become psychotic. For example, Howard Hughes, one of the wealthiest men of history, was a nearly total recluse beset with psychoses and neuroses in abundance. This shows that people do not need just objects and materialism but the human touch. In the movie the "Awakening" Robert De Niro, was suffering from a terrible tremor from the lack of brain dopamine (likened to Parkinson's) but when he danced with a loving caring female his tremors stopped. You could have all the riches in the world and all those things you think you really need to survive and prosper, but there is nothing like a beautiful smile, a handshake, or a hug or a kiss from another individual, or even an intelligent discussion to make you feel like a million bucks!

People provide the real "highs". People need people. However, in today's screwed up, financially unstable (1.4 million bankruptcies 2009), and very scary world, loneliness and alienation are commonplace. Everything would look perfect if we were all living in a vacuum, but instead millions, if their lucky enough not to be homeless, are probably living in glass houses vulnerable to being shattered.

Where love, compassion and friendship are lacking, there is always synthetic chemistry to turn you on to synthetic "highs.' While this might be true for many, there are also those that just like to get high through drugs and do it for no special reason except to "feel good". Hey, it's for the party not for the escape. In whatever way happiness is sought, whether through other people, drugs or sugar-coated placebos, the end result is that an individual strives in his own way to achieve happiness. Frank Sinatra had it right in his song- :"I did it my way!" However this idea of letting people do their own thing has resulted in billion dollar industry –so called drug rehabilitation with its 11,000 or more treatment centers.

[XII] HAPPINESS: A FUNCTION OF DIFFERENT STROKES FOR DIFFERENT FOLKS

The term happiness is a universally accepted description for pleasure, but despite its universality we must recognize that the concept happiness means "different strokes for different folks". While we as homo *sapiens* are basically hard wired in the same way, there is enough evidence from genetic studies that certain ethnic groups have more or less polymorphisms (gene forms) which can effect protein expressions multiple ways. These gene expressions can be visible (color, skin texture hair etc) or non-visible affecting one's emotions, sensibility, intelligence and even proneness to getting "high". Besides differences because of cultural upbringing, our individuality may be tied to our genome. [50] In fact Barr and Kidd investigated the frequency of the DRD2 A1 allele in 381 unrelated people from 16 different populations. On a global scale the frequency of the A1 allele was found to be dramatically different among the populations studied, from as low as 0.09 to as high as 0.75.

No matter how perfectly organized a society might be, individual differences must still be accounted for in dealings with others. As Margaret Mead pointed out in the introduction

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to her 1953 classic *Cultural Patterns and Technical Change* [51]

" Cultural forms, as human beings have so far developed them, while inclusive enough so that most of the human beings who survive within them can live in terms of their institutions and values, are not sufficiently varied and flexible to protect and express all of the individual personalities- each organized whole- in the society. It is to bridge this gap, between culturally provided solutions for the problems of each individual life and the difficulties which many individuals find in using those solutions, that individual psychotherapy has been developed and is now a recognized part of modern culture."

Thus, the word happiness takes on many forms, and can only be defined by each of us in our never ending quest to get it. Happiness is individualized. In very simplistic terms, if an infant could even conceptualize what happiness is, it might be its mother's breast or a bottle of milk.

If you look at a growing child, happiness to this little child might be a toy or a little friend to play with. What is beautiful about children is that their script is not totally formulated, if you believe that premise as presented in *Games People Play* by Eric Berne and Claude Steiner [52]. Have you really taken notice of how kids play? It is beautiful. They shove a little bit, they might push a little bit, and smell a little bit, and they are definitely uninhibited. They're right out there, right in front. Maybe we could learn something. Just maybe many of us need to lighten up, get out there, let loose, and let others live!

In order for our pursuit of happiness to be successful, we need certain provisions as a bare minimum. These provisions or basic needs are very simple. For example, we all need a little food in our stomachs, although it could be poison if we take too much of it. There is also a need to have a few clothes, especially when the cold wind blows, and an adequate shelter to keep us from freezing or getting wet. Then there is the most important basic need that goes by the name of love. Another hard to define word and is in the eyes of the beholder. It is an individual thing. It has many shapes and forms. It is the subject of everyday life –either having it or not having it or trying to get it. Always achieving or looking for a higher love, sometimes missing it when it is right in front of us. Sometimes all the trying is for naught. Is it destiny or is it just a consequence of our actions. Whatever it is, one thing is clear, for most, it absorbs our spirit, our mood, our well-being, except if we turn it off to protect ourselves. The real question worth pondering is not how to get it, but how to keep it.

And there's the rub. Many people, including the affluent in material goods and personal relationships, are convinced that even with the apparent fulfillment of their basic needs, something essential is still missing. That essential ingredient is pleasure. And for millions all they really would like is to free their souls and get lost in Rock' N' Roll and drift away. In fact, most of us always want just a little bit more and some are brave enough to get it. Even against all odds! Maybe happiness is in our genes caressed by our environment. [53].

[XIII] CHEMICAL ORIGINS OF PLEASURE: THE ULTIMATE STATE

There is a basic need in man to achieve pleasure states, and some think that drugs provide a means of getting there. For one person it might be a couple of martinis on the rocks, while another might chew coca leaves. We have not yet learned to curtail or control our abuse of various kinds of pleasure states – whether talking about drinking or smoking pot or gambling, watching T.V., having sex or whatever –sooner or later society is going to have to learn to deal with drugs and pleasure in ways other than by sheer emotional reaction. If we take a step backwards in time, Joel Fort in his 1969 book, *The Pleasure Seekers,* [54] carefully documented that people need pleasure and will actively seek it out. However, there are those who believe seeking pleasure is learned and not inborn. We prefer to think about it as the interaction of both our genes and the environment.

Nevertheless, just the idea of pleasure in its purist form excites neurons, dilates blood vessels, speeds the heart, increases blood flow, makes gastric acid flow, raises the pulse, opens up the pupils, increases saliva and all body juices, tingles the spine, depolarizes muscle, piloerects, and causes a bodily explosion resulting in total loss of control filled with euphoric glow or high.

We are all very familiar with the fact that the brain contains *Poppy –like* material, polypeptides known as endorphines which are opiate-like in biological action and may set off a series of reactions which could lead to a euphoric state not so different from that obtained from ingesting, snorting or main lining opium or heroine. Over the last 30 years of research since they were first discovered, endogenous opioids are key in terms of well-being and pain relief. They interact with opiate receptors in the brain and induce dopamine release by inhibiting the substance GABA [33].

The discovery, in the mid 70's of the opiate-like peptides as well as the earlier findings of opiate receptors in the brain raised interesting questions about the addictive process and the natural innate mechanisms responsible for euphoria. For example what happens chemically when a person has a natural deficiency of these and other brain chemical messengers such as serotonin, and dopamine. Can this sort of deficiency drive some people into seeking another kind of euphorogenic-producing substance to make up for that deficiency? Are imbalances in either the production and/or receptor affinity responsible for certain "abnormal" behavior which we label manic-depressive illness, and/or schizophrenia and /or Reward Deficiency Syndrome? In essence we now believe that certain genes and

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their polymorphisms (gene variants) may either under express or over express either enzymes involved in the synthesis and/or destruction of brain chemical messengers and/or their receptors which will influence one's proneness to addiction. These concepts should provide insights into the notion of "pleasure" as a natural entity as far as being possibly mediated via naturally occurring substances. Is this why we have a poppy field growing in our brain?

Andrew Weil and Winfield Rosen were right on when they wrote the book "*From Chocolates to Morphine*" [55]. Did you know that for example, when you drink alcohol, it is converted in the body to a morphine-like substance called TIQ, which was found in the brain and acts biologically via opiate receptors [56]. This substance has also been found in cocoa and chocolates [57]. So the concept of from "chocolates to morphine, is not merely cute but is scientifically correct, albeit the potency of morphine (in terms of getting you high) being much higher than any Godiva bar. It makes you wonder about how many pounds of chocolate are consumed in the United States alone. Over 3.1 billion pounds of **chocolate**, was consumed in 2001 worldwide. To be facetious, if chocolates and morphine have similar pharmacological properties, would society place a ban on the sale of chocolate because of its addictive qualities. Could you imagine an anti- chocolate campaign or a chocolate prohibition!

Moreover, as stated earlier, the brain chemical dopamine influences how people make decisions; both simple and complex decisions, from what to make for dinner to whether to have children. According to a recent publication when making real-life decisions, dopamine has a role in signaling expected pleasure from those possible future events [42] including the desire for chocolates.

All of this provides people with one of their most frustrating paradoxes. On one hand we all seek fulfillment and satisfaction through the attainment of pleasure states –naturally or synthetically induced. On the other hand, while we all have urges, needs , desires, wants and cravings, all of which could be natural, human beings can easily overload their pleasure circuits through abuse, misusage or simple miscalculation. The real question then is - ***Where do we draw the line? When should society step in? What should be the punishment for overindulgence?***

With due respect, most of us travel through uncharted territories of pleasure and there are many pitfalls hidden along the way. In terms of our sexuality should society condone same sex marriages, clergy accepting sodomy, while (not condoning) making laws against consenting adult prostitution and pornography. Basically, society does not always condone pleasure if it is not a pleasure that is convenient to current social mores. The long arm of the law often interferes with the pursuit and enjoyment of pleasure, drawing pleasure itself from the process. Would it be better to say ***"If it don't kill don't kill it!"*** This is a true dilemma when we consider that over one million people were admitted to a drug rehabilitation treatment facility in 2009 [58].

[XIV] CRIME AT THE GENE

Are genetic factors likely to influence a person to become violent? Many scientists have searched for an answer to this perplexing question. In fact the criminologists of our time have often suggested that murder maybe hard wired into the brain of the serial killer as a form of pleasure.

Advances in our knowledge of the neurobiology of aggression and violence have given rise to rational pharmacological treatments for these behaviors. The main biological systems which are known to be involved are those reward neurotransmitters and include serotonin (5HT), opioid peptides (END), gamma-aminobutyric acid (GABA), and the catecholamines (dopamine [DA] , acetylcholine(Ach) and Norepinephrine [NE]).

14.1. Serotonin

A large body of data has emerged linking aggression in humans with low serotonergic function. Yaryura-Tobias et. al. [59] reported higher levels if aggression in adults with low blood levels of serotonin. Linnoila and colleagues [60] reported that impulsive aggression was associated with low levels of the serotonin metabolite 5-hydroxyindoleacetic acid in the cerebrospinal fluid. Others reported on a Dutch family in which a gene mutation in the monoamine oxidase enzyme (MAOA), resulting in a defect in the breakdown of dopamine, serotonin and noreponephrine, was associated with markedly increased aggressive behaviors in teenagers. [61]. Moreover, Muenlenkamp et. al. [62] has reported that stimulation of the 5-$HT_{1A,}$ 5-HT_{1B} and 5-HT_2 receptors reduces offensive aggression, while defensive aggression is reduced only by stimulation of the 5-HT_2 receptor. In muricidal (murdering) rats, 5-HT was higher in the hypothalamus compared to non-muricidal animals as well as higher levels of 5-HT in the amygdala. The serotonin metabolite 5-hydroxyindoleacetic acid (5-HIAA) was also found to be higher in the hippocampus of the muricidal rats [63].

14.2. Catecholamines

In animal studies Haller et. al. [64] found that enhancing catecholamine function by treatment with alpha –2 adrenergic receptor antagonists increased aggressive responses to aggressive intruders. Further experiments [65] in rodents revealed that tricylics and MAO inhibitors, which increased both DA and NE activity, also enhanced aggressive behavior in these animals. In humans, the NE metabolite 3-methoxy- 4-hydroxyphenylglycol correlated with a positive history of aggressive behavior [66] and a positive correlation between aggression and blood levels of phenylethylamine was also found in humans [67]. Acute isolation –induced fighting in

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mice produced a striking "dose –dependent" increase in K_M and V_{max} for dopamine uptake in mesocirtical nerve endings (synaptosomes) but no significant changes for these uptake constraints in nigrostriatal terminals [68]. Moreover, the dopamine metabolite 3-4- dihydroxyphenylacetic acid (DOPAC) was significantly lower in muricidal rats compared to nonmuricidal animals. The hippocampus of muricidal rats showed significantly higher DA levels, as well as higher levels of the NE metabolite homovanillic acid (HVA) [63]. Breese and associates provide evidence to suggest that lack of brain dopamine during development increased the susceptibility for aggression and injurious behavior by influencing D1 dopamine receptor function [69]. Furthermore, work from Comings *et. al.* [70] shows a strong association between aberrant drug seeking behavior and polymorphisms of the D1-dopamine receptor gene.

14.3. Opioid peptides

The role of opioid peptides has also been studied with regard to aggressive behavior and fighting in animals. Beta-endorphin blocked the development of shock –induced fighting, while Naloxone facilitated it but only when shock induced fighting occurred at a low rate. The beta-endorphin induced reduction of fighting behavior was blocked by naloxone, suggesting opiate induced receptor mechanisms in aggressive behavior [71]. Moreover, Comings et. al. [72] also associated the enkephalinase gene with low amplitude P300 waves, which has been associated with violent offenders (see P300 wave map in a violent subject presented herein).

14.4. GABA

GABA is found ubiquitously in the central nervous system; its function is reducing neuronal activity. Eichleman [73] concluded that GABA stimulation centrally reduces aggression, but some studies showed a significant percent of patients treated with benzodiazepines becoming more aggressive.

14.5. MAOA: is it a crime gene?

Moreover, research on young people in New Zealand and Australia found a link between the MAOA gene, a person's environment during childhood and the likelihood of subsequent violent behavior. The gene MAOA stands for monoamine oxidase, and it codes for an enzyme in the brain that is a sort of a clean–up enzyme, and the role of this enzyme is to clear away excess neurotransmitter (especially serotonin) after the brain has responded to a stress. The gene has two forms, a low activity MAOA form which is found in one-third of the human population, and about two-thirds of the human population has a high activity genotype. In the people with the high activity genotype, it's simply that their neurotransmitter system is more efficient and more quickly returned to a healthy balance after coping with stress, whereas those with the low MAO genotype might suffer more from stress and have difficulty recovering after a stressful event.

While the gene may not predict violence it does associate with boys that had been victims of maltreatment. In this regard, Terry Moffitt et al., found that those boys who suffered maltreatment, had the low MAOA activity genotype, and were in fact, much more likely to become violent as adults. So 85% of them had a conviction record for a violent crime with New Zealand or Australian Police. In fact they were only 12% of the boys in their city, but they accounted for 44% of the crimes that were committed [74].

These and other genetic findings involving the dopaminergic system, support the concept of polygenic inheritance. It is the total number of polygenes (for example, dopamine D2 receptor gene and the dopamine transporter gene) in combination with one's environment that will ultimately lead an individual down that violence pathway.

Serial killers as portrayed in the movie MONSTER are a very good example of how child abuse can impact future behavior [74]. More interestingly, as portrayed the monster had great outbursts of violence while under or even after a stressful event. If DNA tested, would she carry the MAOA low activity gene form and maybe other gene forms in a number of neurotransmitter pathways? Thus, albeit, providing an incomplete gene map of violent behavior, it would have been able to potentially predict her risk for such behaviors including murder. Our laboratory has already published on hypodopaminergic genes and pathological aggression. [75, 76].

14.6. Case studies of violent offenders

The above topographical brain map **[Figure–1]** of a violent substance abusing teenager, is similar for violent substance abuse offenders in treatment at the PATH Medical Clinic over a ten year period. A few such case studies follow:

Case-A

A 52 year –old man guilty of the violent crime of killing his father and mutilating his body was arrested. Following his arrest, The Sheriff's department requested a BEAM map. This person was found to have the typical bi-temporal abnormalities consistently observed in many violent individuals.

Case-B

A 32 –year –old man who attended a prestigious Ivy League school who played football for the school's varsity team. One night after graduating from college, shot his lover in a fit of jealous rage. The man was arrested and spent three years in jail for the shooting. Following his release a beam map showed the

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typical bi –temporal abnormalities, including low voltage p300. Blood test revealed low tyrosine levels.

Case-C

A 28-year old woman with a history of opiate dependence with comorbid alcohol and cocaine use had violent outbursts and rage. As a child she was involved in a skiing accident and, also during childhood, she experienced mood swings. Ongoing analysis of the electrical status of her brain via the beam technique revealed temporal lobe abnormalities.

We are providing BEAM representation of a normal vs abnormal brain depicted in **Figure–1**.

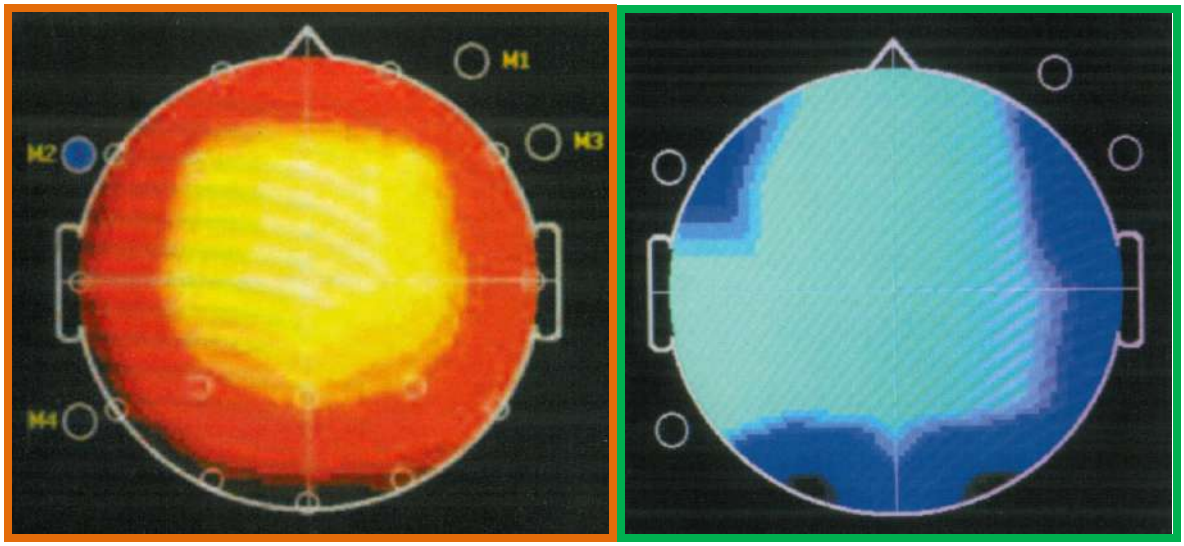

Fig: 1. BEAM image of a healthy brain (Left). The even and symmetrical dispersion of red and yellow indicate a balance in brain chemicals and behavior. **BEAM image of a violent subject's brain** (Right). The large intense area of light blue indicates a severe GABA deficiency, manifesting in the GABA deficient symptoms of rage and violent behavior (with permission from Eric R Braverman, MD –*The Edge Effect)*.

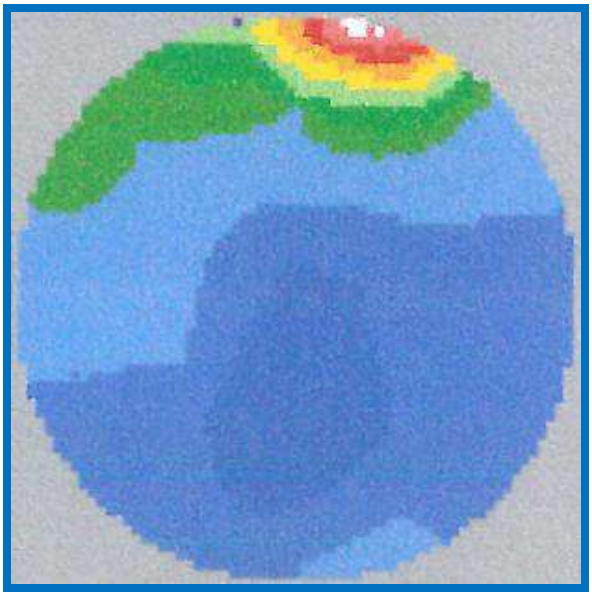

Fig: 2. This BEAM image shows a large amount of light blue, indicating a severe GABA deficiency, manifesting in a GABA deficient symptoms of rage and violent behavior

Figure–2 represents the BEAM of a violent person putatively showing abnormalities in the GABA system. This BEAM representing possible GABA deficiency may result in excessive DA release especially in the amygdale leading to rage and violence.

One major problem is to recognize that when we consider the pathological violence phenotype it may be represented by some combination of a number of behavioral tendencies. In this regard, when we consider reward dependence behaviors an emerging concept called "REWARD DEFICIENCY SYNDROME (RDS)" may help define this complex array of behaviors [36, 77, 78]. RDS broadly defines a common genetic tendency whereby the individual may be predisposed to a number of addictive, impulsive and compulsive behavioral tendencies. It appears that this phenotype is so heterogeneous that it may be not useful. However, the need for homogeneity in the affected phenotype is important not only for population-based association studies but also for linkage analysis. Thus we consider that under the RDS concept there is a remarkable list of behavioral tendencies including: dependence on alcohol, psychostimulants (cocaine), opiates, marijuana, nicotine (smoking), carbohydrates (sugar binging), pathological gambling, sex addiction and even Attention Deficit

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Hyperactivity (ADHD), and aggression and violence, having the same genetic defects under one rubric [36].

While there are poly genes involved, there is close similarity in terms of all of these substances and behaviors induce pre-synaptic dopamine release at the NAc. The common genetic basis may yield more of chance of obtaining homogeneity than if the so called affected probands appeared to be so diverse in their behavioral tendencies (more heterogeneous). In our opinion it would be very difficult to separate alcohol from other drugs as well as other addictive, compulsive and impulsive tendencies such as pathological violence [79]. We as scientists are pressed to answer the very complicated question concerning whether or not genes can be blamed for violence amongst school age killers. "Are some kids simply born bad?" The short answer is yes, as we see from the data presented herein, genes play a role in aggressive and violent behavior. It is our opinion that criminals and or even terrorists may share common gene polymorphisms. Studies at the University Of Wisconsin and others using identical twins raised in different families, who had parallel lives, showed that about half of human behavior (including aggression, sexuality, mental function, eating disorder, alcoholism and drug abuse or generalized RDS) can be accounted for by DNA [80]. Genome –wide scans have shown significant heritability of many genes involved in addictive behaviors [81]. The driving nature in behavior is that very few elements of behavior depend upon a single gene: a complex of genes (polygenic), often across chromosomes, drives most of our heredity-based actions.

Certainly abnormal functioning of these brain systems can be due to specific genetic factors as well as abuse of various psychoactive substances, particularly alcohol and stimulants. In this regard, it has been shown that these individuals may have a reduced number of dopamine D2 receptors [82, 83] associated with the dopamine D2 receptor gene polymorphisms [84, 85] and a high number of dopamine transporter sites [86].

Understanding the interaction of these components and the current literature, has led to some degree of success in the management of aggressive and violent behaviors (the limbic psychotic trigger reaction.), using selective serotonin re-uptake inhibitors (SSRIs), lithium carbonate, beta-adrenergic blockers, anticonvulsants, anxiolytics, typical and atypical neuroleptics, and novel agents such as anti-androgens and serenics (agonists which act on 5-HT1A and 5-HT1B receptors) [87]. The most parsimonious approach may be the utilization of serotonergic and dopaminergic agonist therapy [88, 89]. However, information derived from DNA studies will provide better targeting.

[XV] ARE WE MAKING LAWS AGAINST NATURE?

If indeed we are pleasure –seekers and our brain producers naturally occurring substances with potential euphorogenic properties, then it should follow that people will seek out natural ways to "turn on". Many of us are acquainted with others who seem always bored, or unhappy or sad and nothing seems to turn them on except when they turn to psychoactive chemicals for brief glimpses of paradise. These people turn on to the same phenomenon as the diver who dives 100 feet into the depths of the sea, the skydiver speeding toward the ground, or the daredevil jumping his motorcycle over a river. In every case the resultant effect is one characterized as being pleasurable, filled with gratification of the senses or mind.

While we are cognizant of the need to safe guard our citizens against real harm can we be so bold as to make laws against nature. Are there laws against the sea diver, skydiver, daredevil, soccer player, boxer, teenagers and lovers seeking out love? If not, then why are there laws against the user of psychoactive chemicals? Certainly, there must be laws to protect society, but how to explain other laws which "protect" against not only consenting individuals but victimless crimes?

People will incur costs to punish defectors [90]; punishment, even at personal cost, activates neural reward systems [91]. Instead of activating medial prefrontal cortex, which is engaged in judgments of self in relation to others, Princeton undergraduate students viewing pictures of homeless people and drug addicts preferentially activated insula and amygdala, limbic regions involved in the emotions of disgust [92] and fear [93], respectively. In these extreme forms of prejudice the neural signature implies that the objects of social rejection were perceived as less than fully human [94].

For years we have heard from eastern philosophers concerning the search for natural highs as a way of life instead of seeking unnatural highs through psychoactive chemicals. Research on the subject might reveal one day in the future that when we "meditate" we do indeed 'medicate", a statement predicated on the potential of endorphin-dopamine interactions. In other words; is the Nirvana of life endorphin induced inhibition of GABA brain activity and thus an extraordinary release of neuronal dopamine at the reward site and its subsequent interaction with D2 receptors leading to an orgasmic high? Thus, if this is what is happening under states of meditation, then mediators are getting high, like those in the drug culture, but using "natural" means and methods.

Most recently Jung et al [95] found that meditation as mind-body training is associated with lower stress, higher positive affect and higher plasma DA levels when comparing the meditation group with the control group. Thus, mind-body training may influence stress, positive affect and the sympathetic nervous system including DA activity. These findings should be confirmed by analyzing cerebral spinal fluid to prevent brain and peripheral admixture. This finding takes on even greater importance when coupled with our recent findings of KB220Z induction of an increased alpha increased low beta bands as observed in protracted abstinent psychostimulant abusers using qEEG analysis [96].

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In a related issue, the drug war to some has been considered harmful. In the US, in the past marijuana laws have contributed to a huge increase in the prison population, with vast racial disparities. Recently, the U.S. attorney general has signaled out legitimate medical use of marijuana for special-law enforcement attention and abuse. Our president Obama has suggested non-prosecution of marijuana users. A Time Magazine cover story (November 4, 2002) reported that most Americans do not want marijuana legalized, but do not want users to go to jail either. Public fear of legalization is understandable; it could bring high –powered corporate promotions, as with tobacco-including campaigns to target young people.

Many believe that U.S. society needs but does not have a middle –ground for activities that individual adults can do personally without breaking the law, yet which are officially discouraged and cannot be commercially promoted. Such a middle ground will become increasingly necessary as technology progresses. The potential FDA approval of a Cannabis Tincture, atomizer, is one such example. So we should be thinking about it now!

On the other hand there appears to be a compelling interest on the part of the legal authorities in opiate use or misuse as a means to achieve pleasure states. Evidently, the law focuses on opiates because people utilizing these substances are compelled to lie, cheat, steal and commit other crimes to obtain adequate supplies and in doing so hurt other members of society. When other members of society are effected, the state should make laws against this potential hazard. However, if heroin or other psychoactive agents (or drugs that mimic heroin's effect are easily available at little or no cost to the seeker, the actual damage against other members of society would become very minimal (there would be no money in it) thus there would be no compelling interest for the state and no laws would be necessary. This may be true if we lived in a vacuum and people were interested in loving and caring for people rather than money. That is why any legalization must be carefully considered on a global basis. This is clouded by the hundreds of millions being genetically predisposed to drug seeking behavior [97].

In the words of Harris and Fiske:

"…Even reactions as immediate as disgust to a dirty, unkempt homeless person or an IV-drug-injector can be altered if one plays the role of a soup-kitchen volunteer attempting to feed the hungry, or a social worker leading someone on the path away from drug-addiction" [98].

Ponder this, if drugs induce pleasure states, and if pleasure states are natural, then how responsible or guilty are those people when they turn to artificial forms of euphoria producing substances? In the same way how guilty are we for our sexual deviance if it does not involve hurting others? Are our laws really adequate for dealing with this human reality? Are we making laws against drugs or against Nature (pleasure)? These are the kinds of questions that need to be explored seriously by the physician, by the research scientist by the legal profession, by the courts and by the social biologist.

[XVI] EPIGENTICS A NEW TARGET FOR THERAPEUTICS

Epigenetics encompasses those heritable changes in genome function, occurring without DNA sequence alteration, that involve (a) transference of gene expression patterns over cell generations, (b) alteration of gene expression during cell differentiation, and (c) environment-induced alterations of gene expression. Despite the maintenance of these changes over the cells' lives and even over multiple generations, there are no alterations to the underlying DNA sequence, instead non-genetic factors induce the genes to "express themselves" differently" (up-regulated/down regulated /no change) [99]. As the putative interface in gene-environment interactions, transgenerational epigenetic inheritance is present in widely differing species. [100,102, 93]. Adverse foetal and early life conditions that disturb normal brain development are associated with neuropsychiatric disorders and epigenetic consequences emerging to early expression [103,104]. This early life adversity effecting adolescent and adult behaviour reflects the putative epigenetic mechanisms through which early life environmental influences determine life-long susceptibility to chronic disease states [105, 107]. Specific expression of a disorder (e.g. psychosis) integrates the relationship between adverse events during childhood and the disease state with epigenetic processes. These processes involve the stress regulating functions of the hypothalamic-pituitary-adrenal axis and the neurobehavioral mechanisms through which specific types of childhood trauma may lead to specific types of (e.g. psychotic) experiences [108]. Despite its complexity, the application of treatment drugs that modify the genome, emphasise the necessity for epigenome targeting. Examples are DNA methyl transferase inhibitors and histone deacetylase inhibitors for disorder-related deficits [101,109].

When the action of one gene is modified by the actions of one or several other, "modifier", genes, epistasis occurs. These modifier genes have their phenotype expressed while hypostatic genes have altered/suppressed phenotypes. This difference is an important determinant of disorder propensity. [110] Genetic epistasis offers plausible mechanisms for the etiopathogenesis of neurobehavioral attributes, such as neuropathological impulsiveness, that contribute to neuropsychiatric disorders [110]. Measurable endophenotypes, both as neuropsychiatric concepts and biomarkers, indicate a point on the pathway from gene to disorder. When linked to an expressed abnormality, it is reflected in clinically unaffected relatives, vulnerability polymorphisms, and the cognitive-emotional domains [111]. Taken together, the relative contributions of endophenotypes

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and epistasis in the mediation of epigenetic phenomena may prove essential to diagnosis, intervention and prognosis [112].

[XVII] THE IMPACT OF EPIGENETIC BIONANOTECHNOLOGY ON DELIVERY OF ACTIVE MOLECULES

Bionanotechnology is the key functional technology of the 21st century. The possibility to exploit the structures and processes of biomolecules for novel applications in materials, biosensors, bioelectronics and medical applications has created the rapidly growing field of nanobiotechnology. At the nano level, atoms demonstrate extreme diversity and uniqueness. The term "Bionanotechnology" is a fusion of biology and nanotechnology based on the principles and chemical pathways of living organisms, and refers to the functional applications of biomolecules in nanotechnology. Guided by studying the structure and function of the natural nano-molecules found in living cells bionanotechnology encompasses the study, creation, and illumination of the connections between structural molecular biology, genetics, nutrition.

Bionanotechnology of "biomimetic membranes" describes the current state of research and development in biomimetic membranes for bionanotechnology applications. The application areas in bionanotechnology range from novel nanosensors, to novel methods for sorting and delivering bio-active molecules, to novel drug delivery systems. The success of these applications relies on a good understanding of the interaction and incorporation of macromolecules in membranes and the fundamental properties of the membrane itself.

The biological and physical sciences share a common interest in small structures (ranging from 1 nm to 1 mm). Development of nano-science around new materials and tools (largely from the physical sciences) and new phenomena (largely from the biological sciences) are happening. The physical sciences offer tools for synthesis and fabrication of devices for measuring the characteristics of cells and sub-cellular components, and of materials useful in cell and molecular biology; biology offers a window into the most sophisticated collection of functional nanostructures that exists.

The present situation of biomaterials which are currently in use are vastly different from those of a decade ago. Although implantable medical devices are still immensely important, medical technologies now encompass a range of drug and nano delivery systems, tissue engineering, cell therapies, organ printing and cell patterning, nanotechnology based imaging and diagnostic systems and microelectronic devices. These technologies still encompass metals, ceramics and synthetic polymers, but also biopolymers; self assembled systems, nanoparticles, carbon nanotubes and quantum dots. These changes imply that our original concepts of biomaterials and our expectations of their performance may have to change. It may be concluded that many substances which were not regarded as biomaterials and may now be considered as traditional structural biomaterials. Hence, substances have been engineered and developed to perform functions within health care where it is directly controlled by interactions with cells and tissue components. These include engineered tissues, cells, organs and even viruses.

Conventional imaging paradigms rely on the detection of anatomical changes in disease that are preceded by molecular genetic changes that go otherwise undetected. With the advent of molecular imaging (such qEEG, fMRI and PET) it will be possible to detect these changes prior to the manifestation of disease. Molecular imaging is the amalgamation of molecular biology and imaging technology that was spawned by parallel advances in the two fields. Fundamental to this technique is the ability to directly image biological processes that precede the anatomical changes detected by conventional imaging techniques. The two main strategies for imaging of biologic processes are direct and indirect imaging techniques. Direct techniques use molecules that have specific affinities for targets of interest that can be radiolabeled or otherwise detected on imaging. Indirect imaging uses reporter genes that are coexpressed with therapeutic proteins or other proteins of interest to image vector-transfected cells. This is important in gene therapy already accomplished with cDNA directed D2 receptors in alchol –preferring and cocaine self administration [37, 38]. Optical imaging and nanotechnology paradigms will also prove to be important additions to the imaging arsonal. These principles take on even more importance when one considers the need to provide the human organism with safe compounds at significantly lower dosage to reduce adverse effects and cost [113-116].

[XVIII] CONCLUSION

While it is true that Homo sapiens in evolutionary terms are changing very slowly it is also true that certain genetic traits such as genes that regulate pleasure seeking may be the exception. At this juncture we do not know whether the DRD2 A1 allele is an older gene allele or is it newer than the DRD2 A2 allele. Understanding this will help explain the nature of humans relationship with pleasure seeking and even possible its benefit to survival. Certainly carriers of the DRD2 A1 allele are more aggressive than carriers of the DRD2 A2 allele [79].

In conclusion, we must ask; Who are the people that could just say NO? In this regard it is noteworthy that almost half of the US population has indulged in illegal drug practices. Why do millions have this innate drive in face of the danger of putting themselves in harms-way? Why are millions paying the price of their indiscretions in our jails, in hospitals, in wheel chairs or lying dead in our cemeteries. What price must we pay for pleasure seeking or just plain getting "HIGH"?

While it is true that imaging studies of the brains of people addicted to drugs have helped to clarify the mechanisms of drug addiction we must reflect on the question of how we address

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legally the natural pursuit of pleasure seeking. Moreover these studies and the initial work of Blum and Noble [13] and others have also helped to change the public's view of drug addiction, from that of a moral violation or character flaw to an understanding that pathological changes to brain structure make it very difficult for addicts to give up their addictions.

The frontal orbital cortex abnormalities of addicts create a feeling of need or craving that addicts know is irrational but cannot prevent. Prefrontal abnormalities also make it difficult to override compulsions to take drugs by exercising cognitive control. The main areas affected are the orbitofrontal cortex, which maintains attention to goals, and the anterior cingulate cortex, that mediates the capacity to monitor and select action plans. Both areas receive stimulation from dopamine centers lower in the brain.

A steady influx of dopamine makes it difficult for addicts to shift their attention away from the goal of attaining drugs. It also fastens their attention to the motivational value of drugs, even though these drugs have long stopped providing pleasure. While the release of dopamine may result in ultimate pleasure states its real importance or relevance is that of sought-after goals. Addicts have a hard time turning their attention -- and their actions -- away from the goal of acquiring and consuming drugs. They are caught in a spiral of physical brain changes and the psychological consequences of those changes, leading to further changes.

What is needed is a little understanding from those in power. Should we need to lighten up, look the other way as long as no one gets hurt? Easily said but not easily done. We need a holistic approach to the process of life that mirrors the intuitive forces unleashed in more primitive societies, before civilization took upon itself the role of arbitrator and rule-maker over the seeking of pleasure states.

In his book "The Origin of Consciousness in the Breakdown of the Bicameral Mind", Julian Jaynes inferred that the sense of self-awareness emerged about four millennia ago when the experiences from the right hemisphere blended with linguistic and other related properties of the left hemisphere. The consequences of these insights are consonant with the authors' observations and portend remarkable possibilities. Perhaps the most compelling congruence with Jaynes's insights is in the field of genetics. It has been shown that single point mutations on genes can produce permanent changes in speech production. There is now evidence that point mutations can diffuse within decades throughout entire populations [117]. As Marcel Kuijsten writes in his recent re-examination of Jaynes' work:

"…reflexive rejection of novel concepts is the antithesis of discovery. Science is the pursuit of the unknowns and open-mindedness to contentious concepts…is the optimal environment for discovery" [117]

The complexity of modern society brings down technological barriers between our inner strivings and those elusive pleasures states which seem always to dance just beyond our reach. We need to get in touch with our own identities, grasp the meanings of symbols which lie beyond our current language and mathematics, and once again confront ourselves in the unconscious and nearly forgotten well-springs of our origin. Rather than allow ourselves to become castaways of a genetic legacy which passes us by on the way to some ultimate and unknown fulfillment, it is time for us to pause, to listen to the voices of our primitive past while going beyond to the pleasure states which comprise our ultimate destiny whether god induced or just natures' way [118].

We all have the right to life. However, does that give us the right to take away the life of others, especially, if we only have 120 years to live? Does that give us the right to take away our choices in life? Does that right include getting high, smoking, having sex or just having fun? It does not include bombing the world trade center, or the holocaust or any other human induced tragedy including the killing of new borne girls or clitoral castration.

As to the questions posed herein related to evolutionary genetics and more importantly gene selection we refer to the work of David Comings emeritus Chairman of the department of Medical Genetics, City of Hope National Medical Center, Duarte, California writing in his popular book: " The Gene Bomb" [119]. In one important scenario Comings suggests that while it may be true that genetic adaptations are very slow there may be some exceptions like the Tibetan altitude gene [4].

Let us assume that the a mutant gene called X causes addiction, and that individuals with this X gene drop out of school earlier and start having children earlier than individuals who do not carry the mutant gene. Let us also assume that the average age at birth of the first child of mutant carriers is 20 years, while for those not carrying the mutation it is 25 years. As a result, the mutant form of the gene will reproduce faster, namely every 20 years, while the normal form of the gene will reproduce every 25 years. The ratio of 25/20 is 1.25. **Figure–3** illustrates the results of using one of the equations [119] that calculates the rate at which the frequency of a gene with such a selective advantage will increase over succeeding generations.

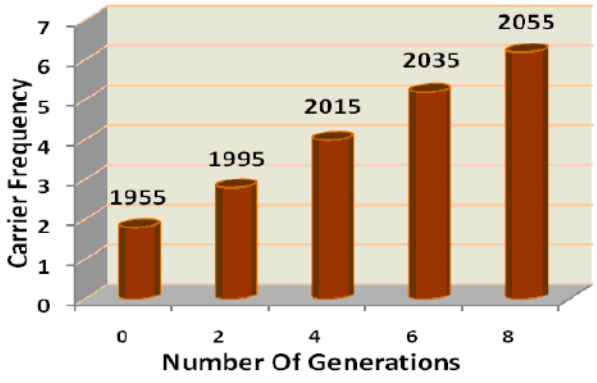

Fig: 3. The rate at which a RD gene that has a 1:25 –fold selective advantage will increase in frequency from generation to generation

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While we must caution against our hypothesis due to many caveats such the complexity of polygenic inheritance rather than one gene –one phenotype including the number of children, number of siblings, number of genes involved, different selective pressures and mathematical considerations, we propose that the carrier frequency of such a gene could double from, for example, from 1995 to 2015 and could have increased 150% from 1955 to 1995. While this gene X may seem to not have any selective benefit one must consider the fact that having low D2 receptors in our current society may confer certain competitive advantages leading (e.g. enhanced aggression, novelty seeking, risk taking) to greater survival.

If this is so then it not unlikely that carrying for example, the DRDD2 A1 allele enhanced prevalence could be responsible in-part for the remarkable increase in RDS globally. In fact it has been shown that the onset of sexual intercourse significantly increases in A1 carriers of the DRD2 gene [120].

Attempts to understand the "High Mind" have eluded the best neuroscientists in the world. In approximately one-third of America, Dopamine is a key genetically induced deficient neurotransmitter resulting in aberrant craving behavior and excessive pleasure seeking. Is it parsimonious that finding ways to enhance dopamine D2 density instead of blocking dopaminergic function may be the best strategy to unlock the elusive addiction riddle and attenuate abuse?

Perhaps for some the answer lies in a leaf, alkaloids found in herbs and plants. PERHAPS THE ANSWER HAS BEEN THER ALL ALONG! We ask are we dealing ourselves enough "***Dopamine for Dinner"*** [121].

FINANCIAL DISCLOSURE

The following people claim a potential conflict of financial interest based on ownership and relationship to LifeGen, Inc patented products: Kenneth Blum, B.W .Downs, Margaret Madigan, Roger Waite, Eric Braverman, John Giordano, and Gwen Bauer, No other author claims any financial conflict of interest.

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REVIEW: MEDICAL LAB TECHNOLOGY

MEASUREMENT OF VITAMIN B_{12} CONCENTRATION: A REVIEW ON AVAILABLE METHODS

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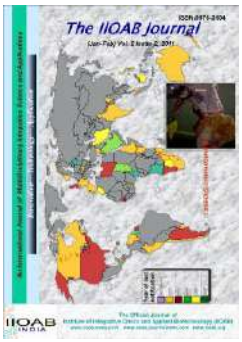
ABSTRACT



Vitamin B12 is a water-soluble vitamin. It is one of the eight vitamins of vitamin B complex, needed for blood and cell maturation. It helps maintain healthy nerve cells and red blood cells, and it is needed in DNA replication. Its deficiency may cause megaloblastic anemia (amidst others health issues). For these and many similar reasons, it sometimes becomes necessary to measure its concentration. This article has carefully reviewed the different methods used for measuring vitamin B12 concentration, and the unique principles involved. The principles, basically, depend on the molecular structure of Vitamin B12 and its reactions with other substances. The methods include microbiological assay and spectrophotometric methods – these are old methods: they were the first available methods, but they are still in use for reference purposes. Another method is electroluminescent (ECL) which involves highly reactive materials. However, inductive-coupled plasma-mass spectrometry (ICP-MS) is a very important method, which is used routinely, even in many research. On the other hand, atomic absorption spectroscopy depends on measuring the amount of energy involved in the reaction; while radioimmunoassay (RIA) is a highly sensitive immunoassay technique. In addition, there are different techniques for separating and preparing samples to be used in the various measurement methods. High-performance liquid chromatography (HPLC) is used for non-validate analyst, while capillary-electrophoresis (CE) that have high resolving power than traditional electrophoresis, which when they are coupled with certain detectors they afford us another principle for measuring this vitamin. Choosing the best method for measuring vitamin B12 concentration depends on many factors – including the type of sample, purpose of the test, necessity of pre-processing, time limitations, cost, sensitivity, specificity.

Keywords: *Electroluminescence; Inductive-Coupled Plasma-Mass Spectrometry; microbiological assay; radioimmunoassay; capillary-electrophoresis; vitamin B12 concentration*

[I] INTRODUCTION

Vitamin B12 is a water-soluble vitamin. It is one of the "B complex vitamins," which play roles in red blood cell formation, nerve cell maintenance, and methyl donation in DNA synthesis. Deficiency of vitamin B12 affects immunologic and hematologic parameter in the body [1].

Human's source of vitamin B12 is of animal origin. It was in 1948 that vitamin B12 was first isolated from liver juice, and it was used in treating pernicious anemia [2]. Vitamin B12 consists of corrin ring (synthesized by bacteria) and cobalt ion; and this cobalt-corrin ring complex gives vitamin B12 its red colouration. Different forms of vitamin B12 are similar in the cobalt central ion, the four parts of the corrin ring and a dimethylbenzimidazole group, but differ in the sixth site which may contain cyano group (CN), hydroxyl group (OH), methyl group (CH3) and/or 5'-deoxyadenosyl group (C-CO) [3,4].

There are several methods to assay and calculate vitamin B12. Some of these methods are used in medical field, and some others in pharmacological studies/investigations. This review

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focuses on some of the weaknesses and strengths of these methods, and aims to identify the best method for measuring the concentration of vitamin B12.

[II] HISTORICAL TECHNIQUES

2.1. Microbiological and spectrophotometric methods

Microbiological method is one of the oldest methods for measuring the concentration of vitamin B12. Information regarding this method has been extensively documented [5].
Ross (1950) was the first scientist to describe microbiological method using *Euglena gracilis var-bacillaris* as test organism. Thereafter there was introduction of Z strain of *Euglena gracilis* so as to shorten the growth period required for the test to as low a five days [6]. Further experiments on measurement of vitamin B12 focused on either changing microorganism test or developing test techniques, such as adding heating step or some substances to the test procedures for converting vitamin B12 to the active free form [6]. Also, several microorganisms were proposed for the microbiological assay. These methods include *Euglena gracilis* tube method, filter paper disc method (FPD), *Escherichia coli* tube method, plate method, *Lactobacillus leishmanii* tube method, bioautographic method, and *Ochromonas malhamensis* tube method [7]. However, *Euglena gracilis* and *Lactobacillus leishmanii* are the most commonly used methods [5,7,8].

Davis et al [6] described a fully automated method for the microbiological measurement of vitamin B12 using chloramphenicol-resistance strain of *Lactobacillus leishmannii* as test organism. Chloramphenicol eliminates the need for sterilization. Using this method, results could be available within 24 hours. This automated method solved the challenge of how to dissociate vitamin B12 from its protein carrier. This was possible by treating the sample with a solution containing glutamic/malic acid [6].

Automated microbiological method was designed to use Mecolab M which is a multi-instrument that provides facilities for sample dilution, reagent addition and mixing, as well as measurement and digital estimation of bacterial growth. It consists of sample preparation unit, autocolorimeter, A/D converter and calculator [6].

Years after, scientists have tried to develop a microbiological method by using microtiter plates. They used chloramphenicol resistance strain of *lactobacillus casei* on serum and red blood cell folates. Then they compared the results with traditional microbiological method. They obtained better results with better intra-assay precision for both serum and red cells (CV% of <5). However, the previous method was more compact, less time consuming, has a lower cost, need smaller amount of sample, and easy to perform in medical laboratories [9].

Microbiological methods are facing difficulties in the assay of vitamin B12, mainly because they are tedious, and time consuming; they have poor precision, and relatively low specificity [10]. Other disadvantage of this method is that whenever the patient serum contains antibiotics, the growth of some assay organisms will be inhibited and false low/negative results would be obtained [10]. Also, *L. leichmannii* assay may give falsely low results in the presence of some antibiotics and antimetabolites. In addition, the *E. gracilis* assays produce falsely low results with sulfonamide and chlorpromazine [11]. The reference normal range of vitamin B12 concentration for which using *Euglena gracilis* method would be good is 200-900 µg/cc [12].

In 1972, scientists started working on photochemistry of vitamin B12 by Pratt [13]. The conversion of cyanocobalamin to hydroxycobalamin takes place readily in the pH range between "3.5 – 6.5" under the action of light. The quantum of the photoaquation reaction of cyanocobalamin is 10^-4 [14]. The photo degradation of cyanocobalamin plays an important role in the stability of vitamin B12 solutions. If the primary photochemical change leading to the formation of hydroxyl cobalamin could be minimized, the photo stability of cyanocobalamin could be enhanced. Spectrophotomery for vitamin B12 measuring was very diverse according to the use of many light spectra like gamma-ray counter spectrophotometer [15]. Ultraviolet (UV) -vis spectrophotometer different types [16, 17] or some reagents were added as 6,7-dimethoxy-1-methyl-2(1H)-quinoxaline-3-prpionyl carboxylic acid hydrazine (DMEQ) to produce a highly fluorescence vitamin B12 derivative [14], and 4,4'-diazobenzenediazoaminoazobenzene (BBDAB) [18]. In UV and visible spectrophotometry, aqueous solutions of cyanocobalamin exhibit maximum UV and visible region at 278nm, 361nm, and 550 nm [19]. However, several factors such as changes in solvent, temperature, and pH can affect the spectrum [20].

Several many colorimetric methods had been reported for the determination of cyanocobalamin. These methods are based on the determination in the content of cobalt which forms complexes with many compounds at different wavelengths. A colorimetric catalytic kinetic method has been developed for the determination of trace amounts of cobalt in vitamin B12 preparation. In acetate buffer (pH.4), cobalt (II) catalyses the reduction of colorless ferric-dipyridyl complex to pink ferrous-dipridyl complex in the dark. The linear determination range is 0-10 mg/10ml cobalt (III) [15].

Finally, application to injections containing vitamin B12 gave results closer to the results obtained by capillary electrophoresis [20]. Spectrophotometric method has low cost and acceptable specificity in comparison with radio ligand assay [20]. However, it is not suitable for complex samples, and the sensitivity is relatively low in such cases – so it is not used routinely [10].

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[III] PRESENT ACTUAL TECHNIQUES

3.1. Electroluminescence (ECL)

Electroluminescence (ECL) is a process in which reaction of highly reactive molecules are generated from stable state electrochemically by an electron flow cell forming highly reacted species on a surface of a platenium electrode producing light [21]. This method uses ruthenium (II)-tris (bipyridyl) [Ru $(bpy)_3$ $]^{2+}$ complex and triproplamine (TPA) and react them with each other to emit light. The applied voltage creates an electrical field that causes the reaction of all materials. Tripropylamine (TPA) oxidized at the surface of the electrode, releases an electron and forms an intermediate which may further react by releasing a proton. In turn the ruthenium complex releases an electron at the surface of the electrode forming an oxidized form of $Ru(bpy)_3^{3+}$ cation, which is the second reaction component for the chemiluminescent reaction. Then this cation will reduce and form $Ru(bpy)_3^{2+}$ and an exited state via energy transfer which is unstable and decays with emission of photon at 620 nm to its original state [21, 22].

The florescence emitted by $Ru(bpy)_3^{2+}$ is detected by standard photomultiplier, and the results are expressed as ECL intensity, which is the measurement of the whole luminescence emitted from the sample [23]. This method employs various test principles (such as competitive principle, sandwich and bridging) for the measurement [22]. The most important one in measuring vitamin B12 concentration is the competitive principle. The competitive principle is applied to low molecular weight molecules. It uses antibodies (intrinsic factor) for vitamin B12 labeled with ruthenium complex. These antibodies are incubated with the sample, then biotinylated vitamin B12 and streptavidin which is coated with paramagnetic miroparticles are added to the mixture. The free binding sites of the labeled antibody become occupied with the formation of an antigen-hapten complex. Then the entire complex is bonded to biotin and streptavidin. After incubation the reaction mixture is transported into the measuring cell where the immune complexes are magnetically entrapped on the working electrode and the excess unbound reagent and sample are washed away. Then the reaction is stimulated electrically to produce light which is indirectly proportional to the amount of vitamin B12 that is measured **[Figure–1]** [22].

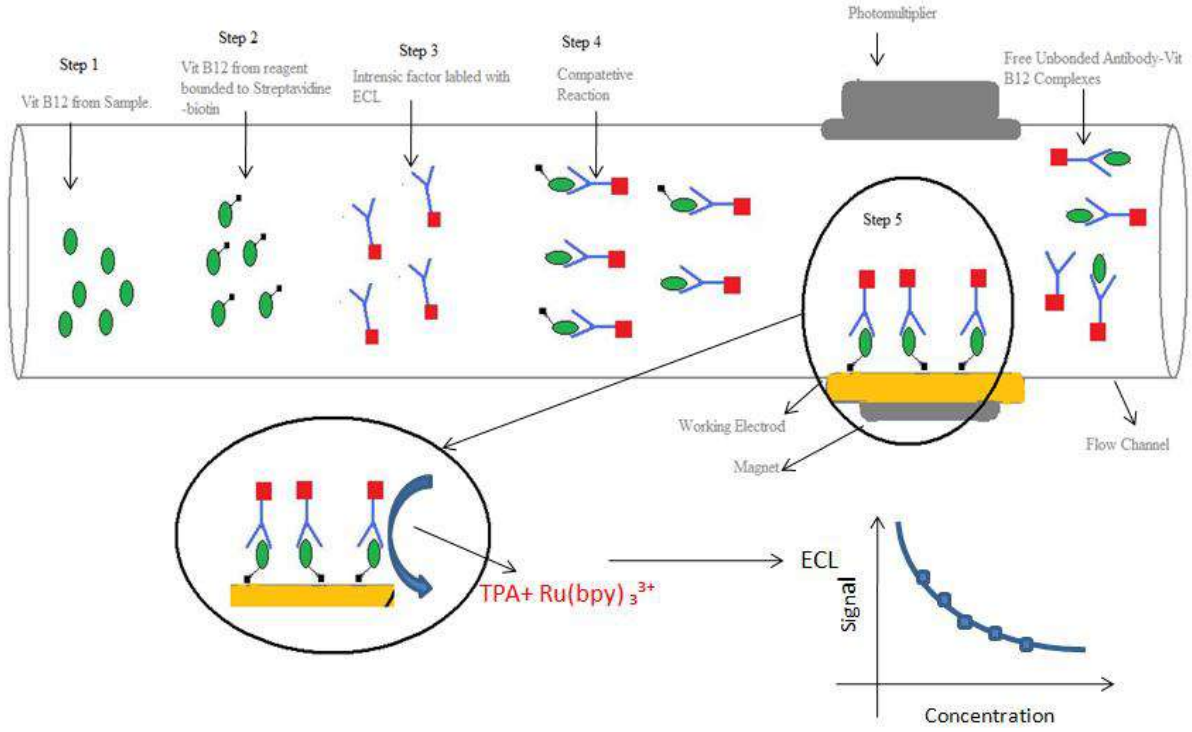

Fig: 1. Electroluminescence method, competitive principle for measuring vitamin B12. **Step 1:** Vitamin B12 from serum sample enters to the flow channel. **Step 2:** Vitamin B12 particles from the reagent are bonded to streptavidin-biotin to help in their attachment to the magnet part. **Step 3:** Intrinsic factor (which acts as antibodies) are bounded to ECL particles to enhance the reaction. **Step 4:** Vitamin B12 from the sample with the particles from the reagent bind to the intrinsic factor. **Step 5:** Only intrinsic factor that is bonded to the vitamin B12 labeled with Streptavidin-biotin particles is attached to the working electrode (by magnetic action) were the ECL reaction will take place and the signal will be measured. The free intrinsic factor with the ones that binds to the vitamin B12 from the sample will be washed away.

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Test sample needed is serum, and the sample duration time is 27 minutes, this test is very sensitive. It can even detect 22 pmol/L (30 pg/ml). It is also very precise (CV% is >10%), and very specific and cross reactivity rarely occurs. This test has high reproducibility, and can be processed easily. Machines used in this technique have extremely long life span with no maintenance costs. An example of such machines is Elecsys 2010 and Cobas e 411. This technique is often used in pharmacological, industrial, clinical and chemical research [24].

3.2. Inductive-coupled plasma (ICP) - mass spectrometry (MS) (ICP-MS)

One of the best methods for the determination of vitamin B12 concentration is mass spectrometry (MS). This is because of its speed, sensitivity, easy (fully automated) and its vast possible application. It is one of the most important instruments for both routine and research applications. In contrast to what its name implies, MS actually measures mass to charge ratio and not just the mass. However, when the charge of all particles (ions) is the same, the mass spectrum plot is simplified to have only mass on the X- axis and the relative abundance on the Y-axis [25].

There are deferent types of MS but they all have three main components in common: an ionization source; mass analyzer; and detector. Ionization process occur in deferent ways in the different types of MS and that is what actually explains the differences between the deferent MS types. Ionization is an important step, and ensures the conversion of the the analyte of interest into gaseous phase ions. The first described ionization source is the electron ionization where the sample must be of low molecular weight, vaporizable and thermally stable. The analytes has to be vaporized and then ionized, and these limited the availability of such method for many biological samples and analytes so there was great need for developmental ionization sources [25, 26]. This lead to the development of electrospray ionization, atmospheric pressure chemical ionization and matrix assisted laser desorption ionization [25].

The first type of ionization source is Electrospray ionization (ESI). It depends on generation of electrons at atmospheric pressure by exposing the sample to different voltage depending on the boiling temperature of the liquid phase sample and the diameter of the inner capillary tube. Most machines with EIS also have additional or optional ionization technique which is Atmospheric Pressure Chemical Ionization or Inductively Coupled Plasma where ionization can also occurs at atmospheric pressure. But these differ from ESI in that sample travels through the different heating zones: when plasma torches it, it becomes dried, vaporized, atomized, and ionized. During this time, the sample is transformed from a liquid aerosol to solid particles, then into a gas, so that they are excited and they gain enough energy to release electrons from their orbits and generate ions. Like ESI and ICP, matrix assisted laser desorption ionization occurs in vacuum where laser irradiation pulsed is the source of ion generation [25, 26, 27].

As Since the ionization sources can differ, there are also several types of mass analyzers. One of the simplest types is “Time of flight mass analyzer” where the velocity of the ions (which depends on the mass to charge ratio) leads to the separation of the ions in different speeds. When fixed potential force them toward the detector, the speed (time) of an ion in reaching the detectors is proportional to its mass to charge ratio – lower ratio is associated with higher velocity. The other type of the mass analyzer is the sector analyzer (magnetic or electric sectors are available). Here the ions are focused toward the detector after they have left the sector, through a split by applying a fixed accelerating potential.

The widely used mass analyzer is the quadruple, especially with gas and liquid chromatography, since it is much smaller, easier, and cheaper than other analyzers. By placing a direct current (DC) field on one pair of rods and a radio frequency (RF) field on the opposite pair, ions of a selected mass are allowed to pass through the rods to the detector, while the others are ejected from the quadrupole. The other ions of different mass to-charge ratios will pass through the spaces between the rods and be ejected **[Figure–2]** [25, 27, 28].

To perform MS one needs to start with pure analyte to be able to use different MS types. So it is important to combined MS with other separation techniques such as capillary electrophoresis, HPLC, gas chromatography, and liquid chromatography, where Cobalamin in human urine and multivitamin tablet solutions can be converted into free cobalt ions in acid medium. The linearity of MS is over the cobalamin concentration range of 1.0 × 10−10 g /mL− to 8.0 × 10−5 g /mL and the limit of detection is 0.05 ng/ mL for both ICP-MS and HPLC-MS. MS is often used in Pharmacology, industries, and in basic research, but not used in clinical field due to its high cost [29].

3.3. Atomic absorption spectroscopy

Atomic absorption spectroscopy is an analytical chemistry technique used for determining concentration of particular metal element in a sample, and it is widely used in pharmaceutics. This technique can be used to analyze the concentration of over 70 different metals in a solution [30]. The discovery of the Fraunhofer lines in the sun's spectrum in 1802 marked the beginning of the main phenomenon behind this technique. However, it was not until 1953 that Sir Alan W (Australian physicist) demonstrated the possibility of using atomic absorption for quantitative analysis [31]. Simply put, atomic absorption spectroscopy has to do with the measurement of the absorption of light by vaporized ground state atoms and then estimating the desired concentration from the absorption. Basically, the incident beam (of light) is attenuated by the absorption by atomic vapor according to Beer’s law [32].

A detector measures the wavelengths of light transmitted by the sample (called the “after wavelengths”), and compares them to the wavelengths, which was passed through the sample (the

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"before wavelengths"). Moreover, a signal processing unit then processes the changes in wavelength, and gives the output for discrete wavelengths as peaks of energy absorption. Since, an atom is unique in its absorption pattern of energy at various wavelengths due to the unique configuration of electrons in its outer shell, the qualitative analysis of a pure sample can be achieved [32]. This, in fact, makes it reasonable for this method to measure the quantity of energy (in the form of photons of light) absorbed by the sample. Using this technique, various metals in organic samples can be analyzed. The basic structure of the machine consists of 4 basic structural elements; a light source (hollow cathode lamp), an atomizer section for atomizing the sample (burner for flame, graphite furnace for electro thermal atomization), a monochromatic for selecting the analysis wavelength of the target element, and a detector for converting the light into an electrical signal **[Figure–3]** [33].

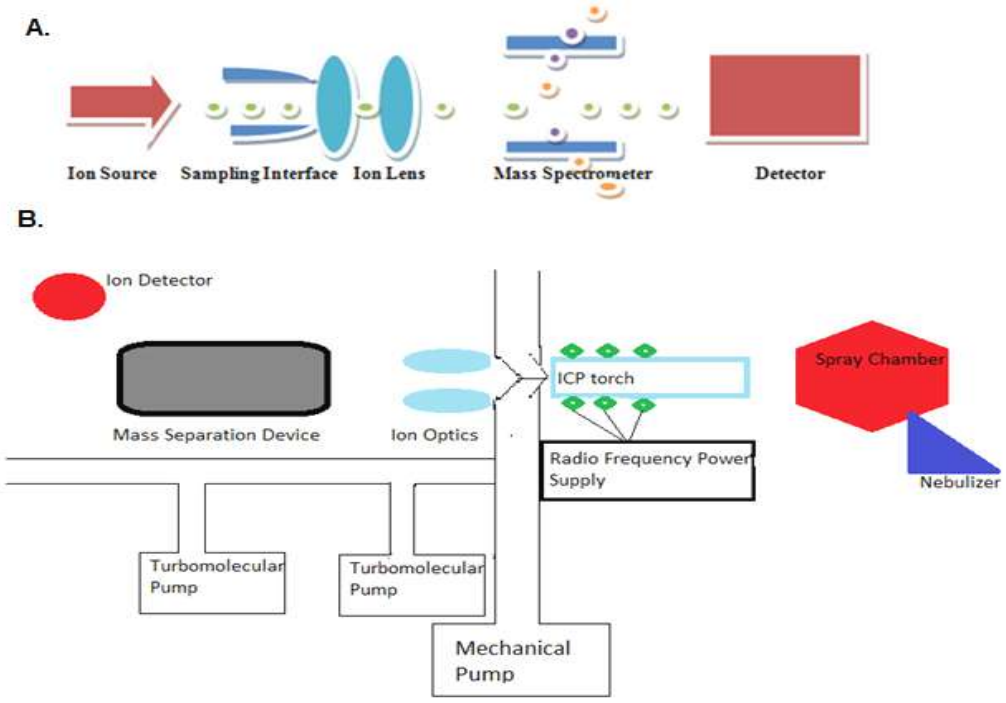

Fig: 2. Inductive-Coupled Plasma-Mass Spectrometry (ICP-MS). A. Mass Spectrometry: Shows the basic components of a typical mass spectrometry. All mass spectrometry shares three main components; an ionization source, mass analyzer, and detector. **B.** Inductive-Coupled Plasma: It serves as the ionization source in some particular types of MS called ICP-MS. The sample travels through the different heating zones and are finally ionized.

Here, the atomizers used are pyrocoated tubes and tubes with centre fixed platforms. In addition, a cobalt hallow cathode lamp is used and a wavelength of 242.5nm could be used for assaying. Argon serves as a protective gas and serum or urine could be introduced into the graphite furnace (GF) directly with equivalent volume of modifiers. H_2O_2 is used to prevent carbon residue formation in graphite tube. The electro thermal atomic absorption correctly and optimally measures Cobalt (and thus, vitamin B12) in serum and urine. It has a detection bound of 0.02 µg/L Co in serum samples with a relative standard deviation of 10-18% [34].

The main advantages of this method is that it has a high sample throughput, it is easy to use, and it has high precision. But the main disadvantages involve its less sensitivity, its requirement of large sample, and the problems with refraction [34]. Another method that is used is the Flame atomic absorption spectrometry. The lowest concentration for quantitative recovery is 4 ng/cm^3 of vitamin B_{12}. The method is used for vitamin B_{12} determination in pharmaceutical samples. It is used in pharmacology, industry, clinical and chemical basic research. [35].

3.4. Radioimmunoassay (RIA)

Radioimmunoassay (RIA) is a highly sensitive laboratory technique used to measure minute amount of substrate (such as, hormones, antigen and drugs) in the body. RIA is a primer immunoassay techniques developed for detecting extremely small concentrations [36]. Berson and Yallow developed the first radio-isotopic technique to study blood volume and iodine metabolism and had used it for the determination of insulin levels in human plasma. Later the technique was adapted for studying how hormones (especially insulin) are being used in the body [35]. This method is so sensitive that it can measure one trillionth of grams of substance per milliliter of blood and only

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small samples are required. These (among other reasons) made RIA to quickly become a standard laboratory tool [37].

RIA is based on the reaction of antigen and antibody in which very small amounts of the radio-labeled antigen competes with endogenous antigen for limited binding sites of the specific antibody against the same antigen. The radio-labeled antigen have been an analogous in biological activity and/or immuno-reactivity to the native antigen. For vitamin B12 we use Modified intrinsic factor (IF) fractions which have R-proteins that bind many porphyrin-ring-containing compounds (i.e., cobinamides) by radio assay with [^{57}Co] vitamin B12 [38].

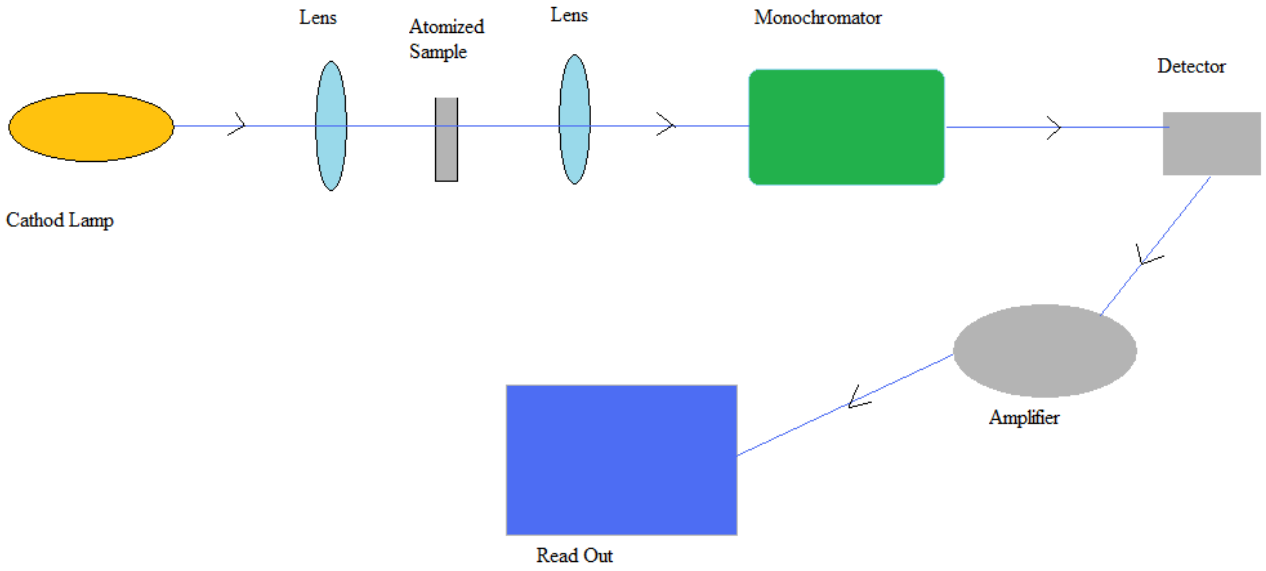

Fig: 3. Schematic diagram of an atomic absorption spectrometer. The basic structure of the machine consists of 4 basic structural elements; a light source (hollow cathode lamp), an atomizer section for atomizing the sample, a monochromatic for selecting the analysis wavelength of the target element, and a detector for converting the light into an electrical signal, amplifier and readout.

Most commonly used radio-isotope in RIA is 125-I. Other emitting isotope such as C14 and H3 have also been used. Some other important aspects of RIA are the use of specific antibody against particular antigen, and the use of pure antigen as the standard or calibrator [37] is attached to tyrosine. These radio labeled IFs are then mixed with a known amount of cyancoblamine, and they become chemically bound to each other. A serum from a patient which contains an unknown quantity of IF is added, so the unlabeled (or "cold") IF from the serum competes with the radio labeled (or "hot") IF for cyanocoblamine binding sites [39].

If the concentration of the "cold" IF increased, more of it binds to cyanocoblamine and this will lead to displacement of the radio-labeled variant, so the ratio of "cyanocoblamine bound to radio labeled antigen" to "free radio labeled IF" is reduced. After that, the bound IF is separated from the unbound ones and the radioactivity of the free IF that remains in the supernatant are measured [39]. The separation of radio-labeled IF bound to cyanocoblamine from unbound radio-labeled IF occurs after optimal incubation conditions (buffer, pH, time and temperatures) [37].

Polyethylene glycol joined with double antibody method is regularly used to separate bound and free radio-labeled IF. Some other techniques in use are the double antigen, charcoal, cellulose, chromatography and solid phase technique [37].
Calibrations or standard curves are formed from sets of known concentrations of the unlabeled standards and from such curves the quantity of IF in the unknown samples can be determined. Improving the sensitivity of the assay is possible by decreasing the amount of radio-labeled analyst and/or antibody, or by disequilibrium incubation format in which radio-labeled IF is added after initial incubation of IF and cyanocoblamine. This technique (just like the others) is supposed to meet the criteria of sensitivity, specificity, precision, recovery and linearity and dilution [39]. For this technique, the precision has been said to be 7.9% for 200 ng/L as the concentration of vitamin B12, 6.6 % for 400 ng/L, and 6.7 % for 800 ng/L. The sensitivity of the assay has also been documented as 37 ± 9 ng/L [39]. RIA is well used in pharmacology, industry, clinical, and chemical research [38].

The principle of radioisotope dilution is based on using unknown quantity of non-radioactive vitamin B12 released from serum to dilute the specific activity of a known quantity of [^{57}Co] vitamin B12. A solution of intrinsic factor concentrate (IFC) with a vitamin B12 binding capacity less than the quantity of added [^{57}Co] vitamin B12 is used to bind a portion of the mixture of radioactive and no radioactive vitamin B12 i.e., to "biopsy" the pool of vitamin B12. The vitamin B12 not bound to IFC is removed by the addition of coated charcoal [40].

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3.5. High-performance liquid chromatography (HPLC)

It is a liquid chromatography used for non-volatile analyst in which the elute do not flow under the force of the gravity but it is derived under a hydrostatic pressure of 5000 to 10000 pounds/square inch through a stainless steel column [41, 42]. The HPLC system uses a mobile-phase pump, a reagent pump, an auto-sampler, a detector and a data system for data processing and system control [43].

The system is a chromatography, in which the eluent is filtered and pumped through the column, then the sample is loaded and injected onto the column and the effluent is monitored using a detector, and the peaks are recorded. The pump of the system must be able to generate high pressure, performing a pulse-free output and deliver flow rates ranging from 0.1 to 10 ml/min [42].

In this method, samples are treated very carefully and the working pH, heating, agitation, centrifugation and filtration are correctly adjusted in accordance with the source of the sample; and the resulting solution is injected into the instrument that does the measurement. he HPLC must be connected to a suitable detector e.g. Micro-mass electrospray mass spectrometer. Its results are often precise, and it is very sensitive with detection limits of 50 nmol/L [43]. An example of this Instrument is Kontron HPLC-system 400. This method is frequently used in pharmacology, industry and basic research [43].

3.6. Capillary electrophoresis

Capillary electrophoresis (CE) was first documented in 1981. It is used to separate peptides. CE have high resolving power than traditional electrophoresis and do not require extremely great skills as high-performance liquid chromatography (HPLC) [44].

CE is quantitative rather than semi quantitative or qualitative, and very small samples (< 10 nL to 1 nL) can be used [43, 44]. The schematic structure for CE is composed of sample vial, two buffer vials (source & destination), capillary, electrodes, high-voltage power supply, detector, and data output. Electroosmotic flow forms the main principle in CE [44]. Generally sample for CE does not require preparations, but in low concentrations biological sample such as serum or plasma, there could be a need for pretreatment to prevent ionic strength and protein-rich matrix from effecting the migration [44,45].
CE can be used for cobalamin separation and for differentiation between different forms of the vitamin B12. The procedure of cobalamin separation is done by using 70 cm capillary length with 20 KV voltage supply, and 9.0 pH tris buffer 25 mM that contain 15 mM sodium dodecyl sulfate as electrophoretic buffer [46].

The main disadvantage of this technique is in its low ability to detect the sample (i.e. low sensitivity) due to the wall of the capillary, which is dissolved by coupling system of capillary electrophoresis-inductively coupled plasma mass spectrometry (CE-ICP-MS). It is mainly used in basic research. [46, 47]

Another source of error that is unique to CE but absent in CE-ICP-MS is the electrokinetic sample injection [45]. On the other hand, no problems are unique to the coupled system method mentioned above. In general controlling the column over loading, calibration to prevent sample aging and facilitate analysis, and buffering according to the sample pH, are important aspects that should always be taken care of while separating vitamin B12 [45].

[IV] DISCUSSION AND CONCLUDING REMARKS

The microbiological method remains the routine method for the determination of vitamin B12 concentration, despite the fact that it is time consuming, and has relatively poor precision, and low specificity. This might be because ECL and radioimmunoassay which are simpler and faster are very expensive - since they require pure intrinsic factor and some special reactants. Also, ECL and atomic absorption spectrometry depends on indirect measurement of the cobalt. On the other hand, Capillary electrophoresis and HPLC methods include the use of UV or visible photometry, atomic absorption and ICP-MS.

Determination of the best way of measuring vitamin B12 concentration would require critical consideration of the required/desired sensitivity and specificity, the available time, and the process of preparation of the sample, as well as cost. Some of the important characteristics of the different methods have been summarized in **Table–1**.

Finally, we should say that cases of serious discrepancies between results of vitamin B12 concentration determined by different methods is highly common. We therefore think that it would be important that every laboratory specifies on it reports the method that had been used when reporting the results of vitamin B12 concentration. This might present clear picture to physician and patient. The reasons for deciding to measure vitamin B12 concentration should also play a crucial role in determining the most appropriate method. For example, the investigator might want to use ECL, RIA or atomic absorption spectroscopy if the results would be used for clinical/medical purposes, while ICP-Ms might be preferred for industrial or pharmaceutical needs or for basic research purposes. More importantly, several advantages and disadvantages of each of these methods govern the choosing of the suitable methods. **Table–2** has clearly summarized some of these.

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Table: 1. Comparison of the Sensitivity of different methods used in measuring the concentration of vitamin B12

Procedure	Sample preparation	Sensitivity
ECL	Serum	30 pg/ml
ICP-MS	Need Preparation	50 pg/ml
Atomic absorption	Urine or serum	20 pg/ml
Radioimmunoassay	Serum	200 pg/ml
HPLC	Need Preparation	68000 pg/ml
Capillary electrophoresis	Need preparation	Depending on the attached method

Table: 2. Advantages, disadvantages and applications of each of the methods used in measuring the concentration of vitamin B12

Procedure	Advantages	Disadvantages	Usage
Microbiological and Specrophotometric	Low cost, Acceptable specificity, Considered as reference method.	Tedious, Time consuming, Poor Precision, Low Specificity, False low results (Antibiotic), Need preparation, Low sensitivity.	Not used routinely, only for basic research.
ECL	Very sensitive, Very precise, Low cross reactivity, High reproducibility and good processability, Very specific, Low maintenance cost for machines uses this method.	Expensive, Only uses serum samples	Used in pharmacology, industry, clinical and chemical basic research.
ICP-MS	Fast, High Sensitivity, Easy (Full automated). Linearity (1x10^-10 g/ml – 8x10^-5 g/ml) , Small sample amount (0.05ng/ml), Use Solid and liquid samples.	Destructive technique, Isobaric, molecular and doubly-charged ion interferences	Used in Pharmacology, industry and basic research, not used in clinical field due to its high cost.
Atomic absorption	Very Sensitive, Fast	Hollow Cathode lamp for each element, Expensive	Used in pharmacology, industry, clinical and chemical basic research.
Radioimmunoassay (RIA)	Highly specific: Immune reactions are specific. High sensitivity: Immune reactions are sensitive	Radiation hazards: Use sra diolabelled reagents. Requires specially trained persons. Labs require special license to handle radioactive material. Requires special arrangements for: Requisition, storage of radioactive material. Radioactive waste disposal	Used in pharmacology, industry, clinical and chemical basic research.
HPLC	It can use different kinds of detectors that determine different sensitivity to the method.	Sample needs preparation and certain conditions.	Used in pharmacology, industry and basic research, not used in clinical approach due to its requirements of certain pH and sample preparations.
Capillary electrophoresis	Quantitative and semi quantitative, Recommended use CE-MS-ICP	Ionic strength and protein-rich matrix affect migration happens in low biological samples. Electrokinetic sample injection Controlling the column	Used in basic research

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REVIEW: VETERINARY MICROBIOLOGY

CASEOUS LYMPHADENITIS: EPIDEMIOLOGY, DIAGNOSIS, AND CONTROL

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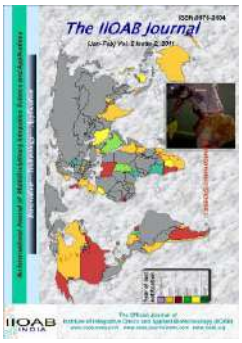
ABSTRACT



Caseous lymphadenitis, caused by Corynebacterium pseudotuberculosis, is one of the most important diseases of sheep and goats, causing considerable losses for herd owners. Due to the chronic and generally subclinical nature of infection, control is difficult and prevalence in animals and herds is high. This review describes the principal characteristics of C. pseudotuberculosis, including pathogenesis, epidemiology and principal manifestations of caseous lymphadenitis, as well as management practices, diagnostic tests and vaccination as disease control tools.

Keywords: ***Caseous lymphadenitis; Corynebacterium pseudotuberculosis; sheep; goat***

[I] INTRODUCTION

Caseous lymphadenitis is a chronic and subclinical disease of sheep and goat of worldwide distribuition, presenting high animal and flock prevalences. *Corynebacterium pseudotuberculosis*, its causal agent, affects sheep and goats, though it can also infect cattle and horses, and rarely, humans; thus, it is considered an occupational zoonosis. The pathogen has been isolated from other species, including pigs, buffaloes, deers, porcupines, llamas, camels and laboratory animals [1, 2].

Distributed throughout much of the world, this disease is found in North and South America, Australia, New Zealand, Europe, Asia and Africa; it causes considerable economic losses, from condemnation of skins and carcasses because of abscesses, to expressive losses in reproductive efficiency, and in wool, meat and milk production. It is the main cause of condemnation of sheep carcasses in slaughterhouses in Australia, one of the world's largest producers of meat and wool [3, 4, 5].

This disease is characterized by abscessing of the lymph nodes; both superficial and visceral. In the superficial form, the peripheral lymph nodes swell and abscess, while in the visceral form there are systemic

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complications that can lead to chronic thinning [6]. *C. pseudotuberculosis* is easily disseminated throughout the herd by normal management practices and by environmental contamination [7].

[II] CLASSIFICATION OF CORYNEBACTERIUM PSEUDOTUBERCULOSIS

Corynebacterium pseudotuberculosis belongs to the genus *Corynebacterium*, family *Corynebacteriaceae*, suborder *Corynebacterineae*, order *Actinomycetales*, subclass *Actinobacteridae,* class *Actinobacteria* [8]. The genus

Corynebacterium belongs to the *Actinomycetes* group, which also includes the genera *Mycobacterium*, *Nocardia* and *Rhodococcus* [2]. Though the species of these genera, also denominated the CMN group, are quite diverse, they have some characteristics in common, such organization of the cell wall, composed principally of peptidoglycans, arabinogalactan, and mycolic acids, and a high proportion of guanine and cytosine in the genome (G + C = 47 - 74%). The CMN group includes many species of medical and veterinary importance, including *Mycobacterium tuberculosis*, *M. bovis* and *M. leprae*, etiological agents of human and bovine tuberculosis, and of leprosy, respectively, and *C. pseudotuberculosis*.

The bacterium *Corynebacterium pseudotuberculosis* is classified into two biovars [9], the biovar Ovis, which mainly affects sheep and goats, causing superficial and visceral abscesses, and the biovar Equi, which mainly affects horses, causing ulcerating lymphangitis of the distal extremities, ventral abscesses of the thorax and abdomen, and furunculosis1. The existence of these two biovars has been confirmed by biomolecular techniques [10, 11, 12, 13].
Corynebacterium pseudotuberculosis is a Gram-positive, nonencapsulated, nonsporing, fimbriated bacterium.14 The cell wall is composed of mesodiaminopimelic, arabinogalactan and corinomycolic acids (lipids), similar to mycolic acid from *Mycobacterium tuberculosis*, but it is not acid-alcohol resistant [14]. The attenuation generated by successive passages is due to thinning of this lipid layer [15].

In stained smears, the rods appear isolated and have pleomorphic forms, from coccoids to filamentous rods, grouped in parallel cells or in a format similar to Chinese letters [16]. According to Collet [17], the microorganism, when removed from culture, does not appear pleomorphic; this was also found for 208 strains of *C. pseudotuberculosis* isolated and identified at the Escola de Veterinária da Universidade Federal de Minas Gerais, obtained from cultures of caseous material collected at a slaughterhouse. The cells are small (0.5-0.6 µm x 1.0- 3.0 µm), facultative anaerobes and generally contain metachromatic granules [14, 17].

Corynebacterium pseudotuberculosis is identified by its morphology, colony characteristics, and biochemical features, mainly carbohydrate fermentation. It produces catalase, sulfidric acid, phospholipase D (PLD) and hydrolyzes urea. Nitrate reduction varies; it differentiates biovar Ovis, which is nitrate reductase negative, from biovar Equi, nitrate reductase positive [9]. In sheep blood agar, incubated at 37°C, cream-colored colonies, with a β-hemolysis zone, are observed after 48 h. It presents a reverse CAMP test, because there is inhibition of β-hemolysis by *Staphylococus aureus* and synergy with *Rhodococcus equi* [14, 16]. In liquid culture, it forms a surface film, though the culture remains clear; this film is broken by agitation, forming flakes [14]. The principal characteristics of *C. pseudotuberculosis* that are important for its identification are shown in **Table–1** [14, 16].

Table: 1. Principal phenotypic characteristics of *Corynebacterium pseudotuberculosis* used for identification

Tests		Carbohydrate fermentation	
Metachromatic granules	+	Starch	-
β-hemolysis	+	Arabinose	V
CAMP	Reverse	Fructose	+
S. aureus	Inhibition	Galactose	+
R. equi	Increase	Glucose	+
Motility	-	Lactose	-
Oxidase	-	Maltose	+
Catalase	+	Mannitol	V
Nitrate Reduction	V	Mannose	+
Methylene Red	+	Ribose	+
Hydrolysis of:		Sucrose	V
Casein	-	Trehalose	-
Esculin	-	Xylose	-
Gelatin	V		
Hippurate	-		
Pyrazinamide	-		
Urea	+		

+: more than 90% positive; v: 21–89% positive; –: more than 90% negative. Adapted from 1Jones and& Collins (1986)[14] and Quinn et al. (2005).[16]

[III] EPIDEMIOLOGY AND ECONOMIC IMPACT

Caseous lymphadenitis is distributed worldwide and generally follows the distribution of sheep and goat herds, though in some regions its prevalence may be under-notified. Dissemination of this disease throughout the world probably occurred through importation of infected animals [18]. From 1996 - 2004, among the 201 countries that reported their sanitary situation to the World Animal Health Organization (OIE) **[Figure–1],** 64 declared that they had animals with caseous lymphadenitis within their borders [19]. These countries are distributed in the Americas (19 of 42 countries), Africa (18 of 51), Asia (11 of 43), Europe (14 of 51) and Oceania (2 of 14) (OIE, 2009). However, the number of countries that have problems with this disease is probably under-notified, because the declaration to OIE is only done by the official sanitary authorities of each country; some countries that have had this disease reported in scientific papers have not made an official declaration, including Brazil.

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Prevalences of caseous lymphadenitis as high as 61% were found in Australia [20]; however, more recent studies indicate a prevalence of 20 - 30%, after vaccination began 5. In the USA, prevalences of up to 43% have been estimated [21], similar to the range of 21 - 36% found among sheep in Quebec province in Canada [4]. In Alberta, also in Canada, vaccination was effective in the reduction of the prevalence of infection.3 In the United Kingdom, 45% of the producers that were interviewed reported abscesses in their sheep [22].

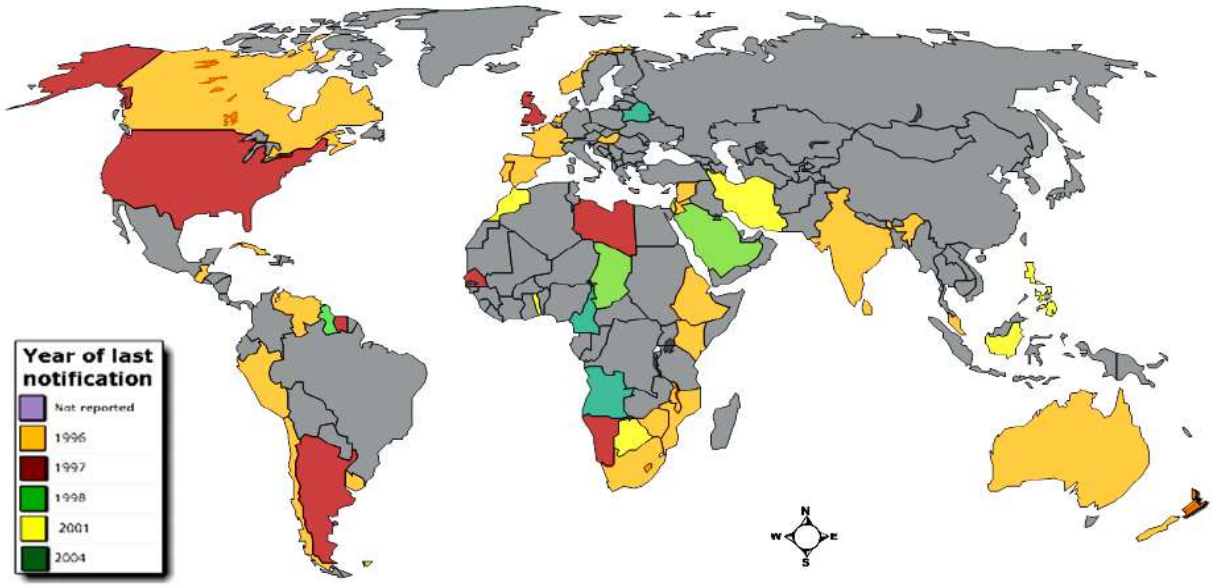

Fig: 1. Map with countries that reported their sanitary situation as a caseous lymphadenitis to the World Animal Health Organization (OIE), from 1996 - 2004

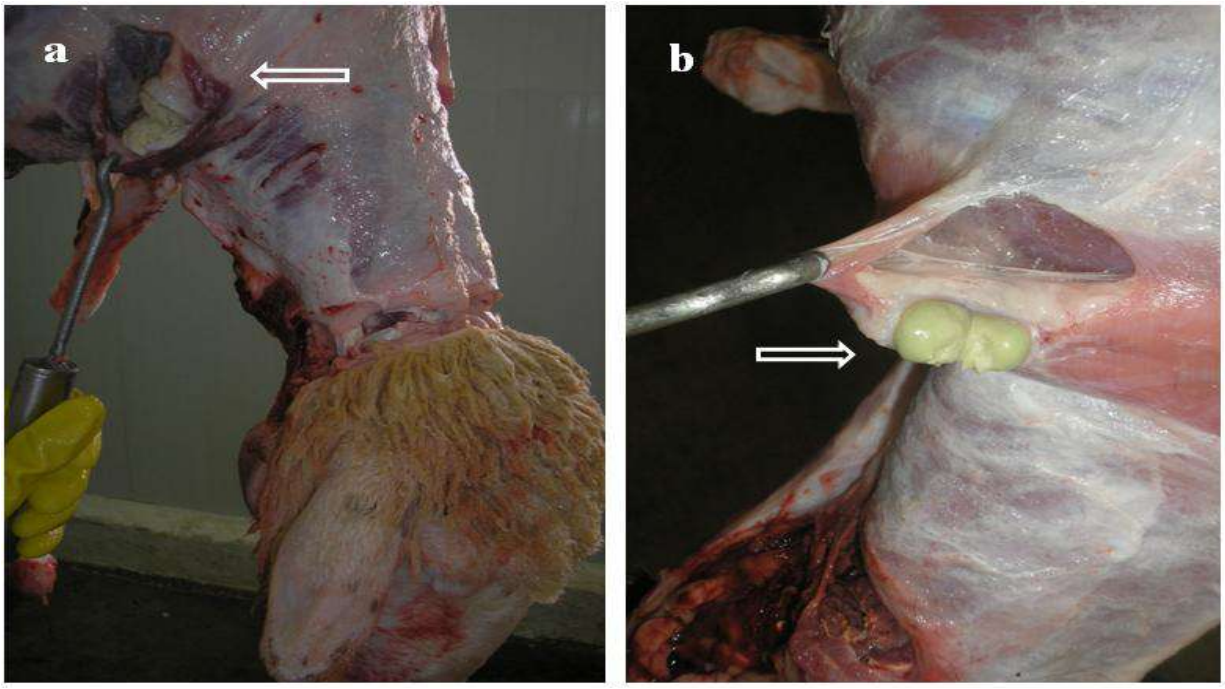

Fig: 2. Condemnation of sheep carcass at slaughterhouse inspection. (a) Pre-scapular lymph node. **(b)** Superficial lymph node. Arrows indicate pre-scapular lymph nodes with caseous material, characteristic of caseous lymphadenitis in federally inspected slaughterhouse

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In Brazil, the first report of caseous lymphadenitis was made by Duport in 1918 [23]. Epidemiological studies have estimated that most Brazilian herds are infected and that clinical prevalence exceeds 30%. In goats, Pinheiro [24] reported 66.9% of animals to have clinical signs of caseous lymphadenitis in Ceará. In Rio de Janeiro, prevalence was reported to vary from 3.6-100% [25] and in a seroepidemiological ELISA study made by our group, in the State of Minas Gerais, we found prevalence figures of 75.8% for sheep [26] and 78.9% for goats [27]. In an ELISA analysis for *C. pseudotuberculosis* in 805 serum samples from sheep from a federally-inspected slaughterhouse in Minas Gerais; we found 377 positive animals, and a high frequency of alterations in the lymph nodes and internal organs **[Figure–2].** This confirms the great economical importance of *C. pseudotuberculosis* infection for the sheep industry due to the high rate of carcass condemnation. Various molecular techniques have been used to type *C. pseudotuberculosis*, including RFLP of chromosomal DNA [11], RFLP of ribosomal 16S DNA [10, 11], ribotyping [10, 11], PFGE (pulsed-field gel electrophoresis) [12, 13] and RAPD (randomly amplified DNA polymorphisms) [28, 29]. Though these various techniques have been useful for separating the biovars Ovis and Equi, the species *C. pseudotuberculosis* has been found to be genetically very homogeneous. The two techniques that have given promising results for typing *C. pseudotuberculosis* strains are PFGE and RAPD.

Pulsed-field electrophoresis was used to characterize 50 strains of *C. pseudotuberculosis* isolated from goats, sheep and horses in the United Kingdom [12]; six "pulsetypes" were observed, which allowed the researchers to determine the origin of an outbreak of caseous lymphadenitis. However, in a study of 36 sheep samples and six goat samples from Australia, Canada, Eire, Holland and Northern Ireland, the same research team reported four different "pulsetypes", with the conclusion that these *C. pseudotuberculosis* strains, both those from sheep and goats, were quite homogeneous [13]. RAPDs were useful in a study of 54 strains of *C. pseudotuberculosis* isolated from horses in four different states of the USA, identifying 10 different genotypes [28]. Also, RAPDs made with other initiators made it possible to define eight genotypes among 61 strains of *C. pseudotuberculosis* isolated from goats in Poland, with a diversity index of 0.539 [29].

The importance of caseous lymphadenitis in Brazil can be estimated by the increase in the participation of goats and sheep in national animal husbandry and its relationship with the economic impact of this disease. Brazil has 16.628.571 sheep and 9.355.220 goats, totaling 25,983,791 animals [30]. The economic losses include decreased milk production, decreased weight gain, reduced value of skins due to scarring, and the cost of the drugs and labor needed to treat superficial abscesses. Losses are increased when the affected lymph nodes are in critical areas (jaw, crural region, udder) negatively affecting chewing, locomotion and milk and meat production; however, economic losses due to this disease have not yet been computed. In industry, losses are due to the lower percent utility of carcasses from affected animals, damage to skins, along with the need for detailed inspection of carcasses. In the Brazilian Northeast, where goat and sheep husbandry are important sources of food and income, the situation is even more critical because of the type of vegetation (spiny) and the low level of schooling of the farmers [31, 24]. It is also becoming more of a problem in the Southeastern, Northern and Midwestern regions, in which this activity is increasing rapidly, negatively affecting the meat-processing industries [26, 32].

[IV] SOURCES OF INFECTION AND FORM OF TRANSMISSION

The main source of infection is infected animals, with or without clinical symptoms; these animals contaminate the soil, water, feed, pastures and facilities with nasal secretions, feces and pus from abscesses that drain spontaneously **[Figure–3]**. Infected animals that do not present clinical symptoms can eliminate the bacteria through their respiratory tract. Evaluation of the coefficients of transmission of *C. pseudotuberculosis* by respiratory tract infection and by pus from spontaneously-draining abscesses, using a mathematical model of transmission, showed that pulmonary abscesses have a small coefficient of transmission, but they are more important for maintaining the infection in the herd (endemic phase) [33].

Transmission can occur through direct or indirect contact or through wounds that come into contact with pus from the abscesses of sick animals [34]. Materials that are used in the management of the animals, such as during castration, identification with ear tags or by tattooing, contact with an uncauterized umbilical stump, and drainage of abscesses, can transmit the agent **[Figure–3]**. Vectors such as insects (especially flies) should be considered in the transmission of the disease, since *C. pseudotuberculosis* has been isolated from the bodies of domestic flies (mechanical vector) and from fly intestines and feces (biological vector). This bacterium has also been isolated from flies contaminated with milk from cows with mastitis in Israel [35, 36, 37]. In horses, flies have considerable epidemiological importance in the dissemination of *C. pseudotuberculosis*, because the higher frequency of infection in this species occurred during periods when there are large populations of flies [11].

Corynebacterium pseudotuberculosis survives long periods in the soil. Through experimental contaminations of soil and of sheep and goat facilities, it was found that *C. pseudotuberculosis* can survive up to eight months at various temperatures [7]. In bedding straw, it can remain viable for three weeks, during two months in hay, four months in shearing stalls and for more than eight months in the soil. This bacterium has been isolated after five months in places where there has been contamination with pus [34] and the concentration of viable microorganisms in the purulent material is estimated to be from 106 to 107 bacteria per gram of pus; consequently, environmental contamination due to a leaking abscess is very high and persistent [38].

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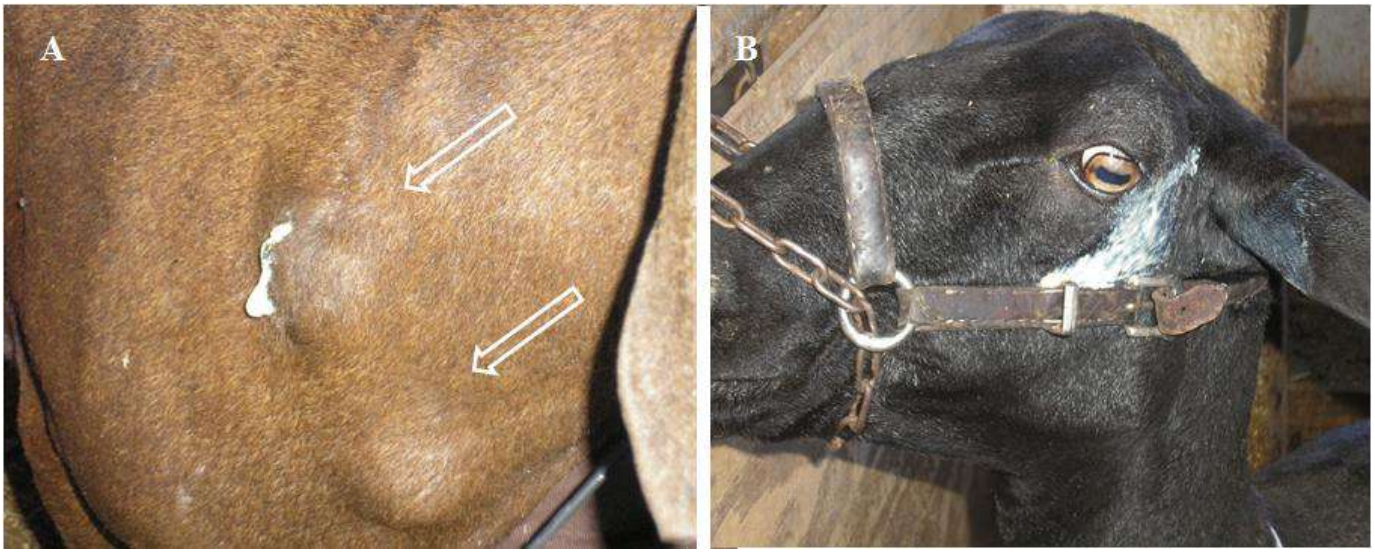

Fig: 3. Environmental and animal contamination with abscess suppurated in sheep. (A) Two caeous abscesses, one spontaneous opened and other closed. (B) Ocular and cutaneous infection of sheep with caseous material

The use of barbed-wire fences or troughs and posts with sharp, cutting edges can cause lesions in the skin of the animals, opening passage for the entry of bacteria [26]. On farms that rear sheep for wool, the equipment and facilities used for shearing can transmit *C. pseudotuberculosis* among animals. Immersion baths immediately after shearing can disseminate the infectious agent, because these solutions can harbor bacteria for up to 24 h [39]. In the Brazilian Northeast, where non-wool sheep predominate almost completely, shearing and tail removal are not common and the sheep are rarely ear tagged [24]; however, the bacteria can penetrate through the respiratory system, transcutaneously or through skin wounds caused by the caatinga vegetation of this region [31].

Goat and sheep meat producers tend to make few periodic inspections of their herds because of the extensive type of rearing system, in which they do not identify individual animals, arguing that these animals are slaughtered within a short time interval. Conversely, goat milk producers tend to identify animals individually and are more likely to detect abscesses during daily contact, favoring the control of caseous lymphadenitis in these herds; this is proved by the fact that 103 (36.3%) of the 284 goat farmer interviewed in Minas Gerais, Brazil, have reported this disease in their herds, while only 13 (6.1%) of the 213 sheep farmers state the same [40]. In this State most goat herds are for milk production, while most sheep flocks are for meat production [40].

Corynebacterium pseudotuberculosis is sensitive to common disinfectants, such as hypochlorite, formalin and cresol; however, the surfaces should be cleaned before disinfection, because organic matter interferes with the action of these agents [41]. Iiodine is recommended for chemical disinfection of wounds in order to reduce bacterial transmission after surgical draining of the abscesses [42].

[V] PATHOGENICITY AND VIRULENCE FACTORS

Corynebacterium pseudotuberculosis is a facultative intracellular bacterium, multiplying within macrophages and surviving the action of phagolysosomic enzymes, because of the external lipid layer of the cell wall [2, 18]. After penetrating into the host, which generally occurs through the oral, nasal and ocular mucosa, or through skin wounds, the agent disseminates freely or within macrophages, mainly through the afferent lymphatic system, to local lymph nodes and internal organs. This process depends on the ability of the agent to infect macrophages, resist phagolysosomes and kill cells, liberating new bacteria and causing necrosis [43]. Three minutes after intraperitoneal inoculation in mice, phagocytic vacuoles are observed; after an hour, 60-80% of the goat macrophages contain bacteria, and two hours after inoculation, acid phosphatase is present in the vesicles containing the bacteria [44]. A strong local reaction occurs four hours after challenge in sheep [45], and a few hours later macrophages are degenerated and polymorphonuclear cell infiltrates containing bacteria are seen [46, 44, 47]. A day after experimental cutaneous infection, microabscesses develop in draining lymph nodes, and pyogranulomas are formed three to 10 days post-infection [48, 49, 6].

The lipid cell layer of the bacteria is pyogenic, but not immunogenic. This same layer makes phagocytosis of the bacteria difficult, increasing its virulence (cytoxicity), and survival inside macrophages; abscesses form through the release of lysosomal enzymes1. Besides participating in pathogenicity,

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mycolic acid appears to be important for the survival of this bacterium in the environment [50].

Phospholipase D (PLD) increases vascular permeability and bacterial survival in the host. It is important for the dissemination of the bacteria from the location of the primary infection (local lymph node) to other organs (lungs, regional lymph nodes, mesenteric limph nodes, etc.), because it lyses mammal cell membranes, rich in phospholipids, causing microhemorrhages and vascular lesions, withincreased vascular permeability [2].

[VI] IMMUNE RESPONSE

Immunity against *C. pseudotuberculosis* is complex and involves cellular and humoral immune responses [51]. Studies point to a greater cellular immune response, chiefly a Th1 response, because of the facultative intracellular nature of the microorganism, with production of gamma-interferon (IFN-γ) and other cytokines that are important for controlling infection [52, 53, 54]. The humoral immune response is observed to present, from 6 to 11 days post-infection, a low production of IFN-γ, which significantly increases thereafter [55]. Inflammatory cytokines, such as TNF-α and IL-6, are mainly produced at the site of inoculation, while T cell-associated cytokines, such as IFN-γ, are chiefly produced in drainage lymph nodes [47].

[VII] CLINICAL SIGNS

Caseous lymphadenitis in its superficial form is characterized by infection of external lymph nodes, such as the submandibular, parotid, pre-scapular, subiliac, popliteal and supramammary lymph nodes, while the visceral form is characterized by abscessing of internal organs, such as lungs, liver, kidneys, uterus, spleen and internal lymph nodes, such as the mediastinal and bronchial lymph nodes. These two forms can coexist; however, other less common sites can be involved, such as mammary gland, scrotum, the central nervous system and joints. Internal abscesses are normally associated with weight loss and weakness, known in sheep as thin-ewe syndrome. The mature abscesses easily leak through fistulas, releasing purulent whitish-green discharges into the environment or into the affected organ. Abscesses usually recur, months or years later, in the same animal, due to the failure to eliminate the infection [1]. In some cases, infections produce few characteristic clinical signs, and a post-mortem examination becomes necessary for diagnosis; this makes it difficult to obtain objective data about disease prevalence [38].

Differences in the place of the abscesses between sheep and goats have been reported, the visceral form being more frequent among sheep and the superficial form among goats [7]. External abscesses in the lymph nodes of the head and neck are more common in goats, while the subiliac and pre-scapular lymph nodes are more commonly affected in sheep [7, 42]. Differences in the appearance of abscess content have also been reported between sheep and goats; in sheep the contents have a laminar form when cut, similar to the layers of an onion, caused by the formation of layers of fibrous tissue and thick caseous material, while abscesses in goats have a thin and pasty exudate [7]. However, onion-like abscesses were not always present in sheep. Sheep carcass inspection at a federally inspected slaughterhouse in Minas Gerais, Brazil, showed that most of the abscesses in sheep were located in the head and neck lymph nodes and their content was essentially pasty. Isolation of *C. pseudotuberculosis* from these materials confirms the infection status of the animals. It is possible that older abscesses become more consistent, with a tendency towards fibrosis and calcification, progressing to an onion-like appearance, independent of animal species.

In horses, there have been reports of abortions and cases of mastitis associated with visceral abscesses.16 In Israel, this bacterium was isolated from subcutaneous abscesses in milking cows; which could occur in outbreaks and cases of mastitis, affecting the whole mammary gland, resulting in total loss of milk production [56].

[VIII] CLINICAL AND LABORATORY DIAGNOSIS

Abscesses in goats and sheep are very suggestive of caseous lymphadenitis, especially if animals of the same lot have similar clinical signs, however bacterial isolation is necessary to identify the causative agent, since other bacteria such as *Arcanobacterium pyogenes, Staphylococcus aureus* subsp. *anaerobius*, *Actinobacillus licheniformis* and *Pasteurella multocida*, can be found in abscesses [57]. In animals with respiratory problems, a thoracic X-ray can reveal masses in the pulmonary parenchyma and lymph nodes; which also must be confirmed by culture of tracheal washes [58].

The use of aspirating puncture with a fine needle in the diagnosis of *C. pseudotuberculosis* was evaluated [59]. It proved to be easily performed, to have a low cost and to cause little damage to the tissues when compared to histopathology. It allows presumptive cytological diagnosis of the infection, before the affected lymph nodes abscessed, aiding in early adoption of prophylactic measures for the rest of the flock.

Gram and Giemsa staining can be used for cytological identification of the microorganism. Although Gram staining is not primarily indicate for staining tissues, the bluish color taken on by *C. pseudotuberculosis*, in contrast with the reddish color of the cellular and inflammatory material from the aspirated lymph nodes, helps in the identification of the infectious agent [6].

In order to make a definitive diagnosis of caseous lymphadenitis, the agent should be isolated from purulent material from abscessed lymph nodes samples from live animals. Besides aspirating puncture, the material can be obtained by excision after trichotomy and careful antiseptic cleaning of the skin [17, 42]. It can also be collected at necropsy or during slaughter, when internal abscesses, affecting the liver, lungs, intestine,

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kidneys, internal lymph nodes and other tissues, become accessible [60].

In the laboratory, after isolation, the identification of *C. pseudotuberculosis* is done by its morphology, staining characteristics, profile and fermentation of various carbohydrates [14]. The main phenotypic characteristics of *C. pseudotuberculosis* used for identification are shown in **Table–1**.

Various diagnostic techniques have been developed for caseous lymphadenitis in goats and sheep, such as serological neutralization for antitoxins, immunodiffusion in agar gel, indirect hemagglutination, complement fixation and hypersensitivity tests [25, 1, 18].

Immunoenzymatic tests (ELISA), using bacterial cells, toxins and secreted proteins of *C. pseudotuberculosis*, such as PLD [61, 62, 63, 64], have been reported to be effective in caseous lymphadenitis control and eradication programs. Indirect ELISA based on secreted proteins has shown a diagnostic sensitivity and specificity of 93.5% and 100%, respectively, in the diagnosisof caseous lymphadenitis in small ruminants [63].

Detection of INF-γ by ELISA, an indicator of cell-mediated immunity, has been used for diagnosis of infection by *C. pseudotuberculosis*, with a sensitivity of 91% and a specificity of 98%, demonstrating its potential for use in caseous lymphadenitis eradication programs [51, 65].

Molecular techniques have also been used for the diagnosis of caseous lymphadenitis. Polymerase chain reaction (PCR), used to identify *C. pseudotuberculosis*, is an alternative to conventional diagnostic methods, with the advantage of being faster and more specific [66]. Multiplex PCR based on amplification of the genes 16S rDNA, rpoB and pld, presented 94.6% diagnostic sensitivity, for *C. pseudotuberculosis* isolates as well as for clinical material [67]. It facilitates the diagnosis by differentiating C. pseudotuberculosis from other pathogens present in abscesses, chiefly C. ulcerans [67].

Recently, the genome of two *C. pseudotuberculosis* strains isolated from goats and sheep has been sequenced by a Minas Gerais Genome Network and Pará Genomic and Proteomic Network. The genomic data will help to identify new specific targets, useful in the diagnosis as well as in the development of drugs and vaccines and in the understanding of *C. pseudotuberculosis* pathogenicity mechanisms.

[IX] DIFFERENTIAL DIAGNOSIS

Pyogranulomatous lesions, such as found in actinobacillosis, tuberculosis and superficial abscesses caused by *Staphylococcus aureus* and *Actinomyces pyogenes*, must be differentiated from caseous lymphadenitis [17]. The superficial form of the disease should also be differentiated from submandibular edema caused by parasites, Fasciola hepatica and *Haemonchus sp*., salivary cysts, lymphosarcoma and subcutaneous inoculation of vaccines. The debilitating visceral form can be clinically similar to chronic parasitism, thinning due to abnormal waste of teeth, alveolar periodontitis, malnutrition and chronic diseases, such as pulmonary adenomatosis, neoplasias and scrapie [17].

Pneumonias caused by *Mycobacterium bovis, Pasteurella haemolytica, Pasteurella multocida* or ovine progressive pneumonia, due to Maedi-Visna virus infection, can make the diagnosis of caseous lymphadenitis even more difficult [58].

In sheep, orchitis and epidydimitis caused by *C. pseudotuberculosis* needs to be differentiated from similar lesions caused by *Brucella ovis, Actinobacillus seminis, Histophilus ovis* and *Pasteurella spp* [17, 68].

[X] TREATMENT

Treatment of affected animals consists of the drainage of abscesses, followed by cleansing and chemical cauterization, usually with 10% iodine, or even removal of the affected superficial lymph nodes [69]. Although it is an important control measure, this procedure might not be as effective as expected due to the presence of internal abscesses. Drainage of the abscess should be done in a way that avoids environmental contamination, with disinfection of the surgical material before and after the procedure, and all of the disposable materials should be incinerated and buried, including plastics and paper used to cover the area.

Another treatment option is antibiotic therapy, which is not very efficient, even though *C. pseudotuberculosis* is sensitive in vitro to almost all antibiotics that have been tested. The intracellular location of the bacteria and the formation of biofilm in natural infections reduces drug efficacy, making antimicrobials inefficient under these conditions [7, 70]. The inefficacy and high cost of antibiotic treatment make it an inviable option for herd-level disease management.

[XI] CONTROL AND PROPHYLAXIS

An effective program for the control of caseous lymphadenitis should be based on clinical inspection and periodic serology of all animals in the flock, which includes recently-acquired animals and those that return to the herd, culling the ones that have clinical signs or that are serologically positive. Once infected, an animal hardly eliminates the *C. pseudotuberculosis* [71]. The main source of infection for a flock is introduction of infected or abscessed animals into a herd, which results in a high frequency of abscesses after two or three years. This stresses the importance of employing biosecurity procedures in all flocks, chiefly during the introduction of animals.

Measures designed to reduce the environmental risk of wounding should also be adopted, such as the use of smooth wire fences, troughs and facilities without sharp edges, disinfection of surgical, ear tagging and shearing instruments, systematic use of

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individual disposable needles, effective control of insects, and disinfection of newborns' navels and any other wounds with 10% iodine. Although it is not recommended to be applied to swelled lymph nodes because of its irritating and caustic action on tissues (skin, mucosa and lungs), 10% formaldehydeshould be used for disinfection of herd facilities [7, 1, 18].

All control programs should be based on sanitary education of herd owners and technical personnel, otherwise success will be compromised. Information about losses throughout the production cycle, as well as concerning the zoonotic potential of *C. pseudotuberculosis*, should be supplied to the people who work with the herds directly or indirectly, reinforcing their importance in the success of the control program.

Control measures vary with the prevalence of infection. In countries free of this disease, importation should only be permitted from herds that have been certified free of caseous lymphadenitis for three years, all animals should be tested by ELISA before importing and they should initially be placed in quarantine. In countries with low disease prevalence, the clinically affected animals should be separated and submitted to ELISA testing, lambs and kids should be reared away from their mothers, and installations and equipment should be well disinfected. In countries with a high incidence, rigorous sanitary measures should be implemented, associated with vaccination [7, 17].

Disease eradication can be achieved in endemically-infected herds by initially discarding all animals that have clinical signs and those that are positive in serological tests [6]; however, this is difficult to accomplish because of the rapid dissemination of the agent within the herd and the difficulty in identifying animals that have a subclinical form of the disease [60, 66].

[XII] VACCINATION

Given that caseous lymphadenitis treatment is ineffective and expensive, the best strategy for control and prevention of the disease is immunization, as it was observed in countries with high prevalence of infection [5]. The vaccines commercially available have different relevant features that should be considered on their use. Not all of the vaccines licensed for use in sheep have the same efficiency in goats, and normally it is necessary to adjust the vaccination program to the flock conditions. Also, the protection provided by vaccination is only partial, as external and internal abscess development can still occur [1].

The principal component of *C. pseudotuberculosis* used in the formulation of vaccines is PLD. The rationale for its use as a vaccination antigen is the good rates of protection obtained after immunization of goats and sheep with this toxin. Most of the commercial vaccines against *C. pseudotuberculosis* use inactivated PLD associated to antigens of other pathogens, such as *Clostridium tetani, Clostridium perfringens* type D, *Clostridium novyi, Clostridium chauvoei* and *Clostridium septicum*, along with some vaccines that are associated with the endectocide moxidectin. Such a formulation is the basis of the Glanvac vaccine (Vetrepharm, Inc London), licensed for use in sheep and goats in Canada, Australia and New Zealand and the Biodectin vaccine (Fort Dodge Austrália PTY LTD), also licensed in Brazil for use in sheep.

The Glanvac vaccine has been evaluated in various countries [72]. Vaccination of sheep and goats with Glanvac resulted in protection against experimentally-induced infection with *C. pseudotuberculosis,* evidenced by a decrease in the number of lesions [73]. Another commercial vaccine that has been evaluated, Caseous D-T (PBS Animal Health, USA), has two formulations, one that only contains toxoids (clostridial and from *C. pseudotuberculosis*) and another that is a combination of clostridial toxoids and the bacterium *C. pseudotuberculosis*. Preliminary results indicate that this second formulation confers better protection against experimental infection than the first, reducing the number of internal and external lesions [74]. The use of PLD toxoid for the immunization of goats can have some negative consequences, including reduced milk production, fever, ventral edema, ataxia and convulsions; therefore, recommendations for its use in this species should be made with restrictions [1].

The partial protection provided by immunization of goats and sheep with commercial vaccines is associated with the type of immune response elicited. Protection against *C. pseudotubeculosis* is mainly dependent on immune response that involves INF-γ production and cytotoxic T-cells. A humoral response alone is insufficient to protect the animal, and a good cellular response is not achieved with inactivated vaccines [75].

Hence, various attempts have been made to obtain an attenuated vaccine that is effective against caseous lymphadenitis [76, 52]. Attenuation can happen naturally or through manipulations using temperature, chemical and genetic (recombinant) agents. With this type of vaccine strategy, the microorganism maintains its capacity to replicate, mimicking natural infection and producing humoral and cellular responses. Also, this is the type of vaccine that confers the best and longest-lasting immune response, due to its similarity to natural infection [75]. Techniques such as deletion of multiple genes involved in virulence, and insertion of fragments that interrupt these genes in the pathogen, practically eliminate the risk that the pathogen can revert to its virulent form [77]. Live vaccines that have been attenuated in the laboratory (recombinants) usually have the PLD gene as a target for attenuation, because of its importance as a virulence factor [78].

In Brazil, the Bahia State Agency for Agricultural Development (EBDA) developed a vaccine based on strain 1002, a naturally attenuated strain, which is currently commercially available. It stimulates significant protection levels, 83%, in vaccine trials; however, immunization still presents collateral effects, such as local reactions, and field trials have not been as successful as the initial vaccine tests, presenting highly variable protection levels [79]. Another attenuated live vaccine, LinfoVac (Laboratórios Vencofarma do Brasil Ltda), licensed for use in sheep and goats, is also currently available in Brazil. The results obtained in the

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field with these attenuated vaccines demonstrate the need to develop a more effective and safe vaccine [75].

[XIII] CONCLUSIONS

Caseous lymphadenitis continues to be an important challenge for sheep and goat industries, limiting their profitability. The intense market and movement of small ruminants, without the necessary biosecurity measures, are important obstacles to the control of caseous lymphadenitis, maintaining its prevalence at high levels, which indicates that specific control measures must be adopted. There are various difficulties affecting the development and application of effective diagnosis and more effective immunogens need to be made available as vaccines. Thus, great efforts need to be made by all players in sheep and goat industries to control this awful disease.

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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RESEARCH: ECOLOGY AND ENERGY RESOURCES

ANALYSIS OF FACTORS AFFECTING SUSTAINABLE COMMERCIAL FUELWOOD COLLECTION IN DAWADAWA AND KUNSU IN KINTAMPO NORTH DISTRICT OF GHANA

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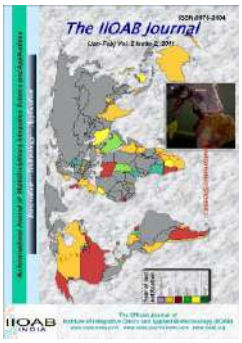
ABSTRACT



This paper examines factors affecting sustainable commercial fuelwood collection in the Kintampo North District of Ghana for the purposes of sustainable woodland management and fuelwood collection. Over dependence on fuelwood collection for livelihood by the rural people in Kintampo North District leads to over exploitation of the woodlands in the area. This situation is a source of concern to managements of woodland and traditional energy sub-sector in the country. Biophysical and socio-economic factors contribute to woodland management in diverse ways: by hindering the exploitation of woodland thereby facilitating sustainable fuelwood collection; and by promoting exploitation of woodland. Focus group discussion was employed to identify factors affecting fuelwood collection in Dawadawa and Kunsu communities of Kintampo North District. Pair-wise comparison was used to rank the factors. Participatory mapping was used to map fuelwood collection sites for relating the collection sites to biophysical factors. Large tracks of land have been exploited at Dawadawa compared to Kunsu, mainly due to the type of land tenure system. Land tenure and low producer price of fuelwood were ranked first in Dawadawa and Kunsu respectively among the factors affecting commercial fuelwood collection. Current collection sites are over 24km and 10km respectively from settlements in Dawadawa and Kunsu. The land tenure system practised in Kunsu promotes effective management system for sustainable fuelwood collection in the Kintampo North District of Ghana; which can be adopted in the other districts of Ghana.

Keywords: *Fuelwood; firewood; energy; woodlands; commercial fuelwood collectors*

[I] NTRODUCTION

Fuelwood (FW) plays an important role in the socio-economic lives of more than two billion people globally [1]. More than 1.8 billion m^3 of fuelwood is used globally and it constitutes about 7% of the world's total primary energy consumption with 76% of it being used in developing countries particularly in Africa [1, 2]. In developing countries such as Ghana, fuelwood is a major source of domestic energy. In the rural communities it is used for cooking, general heating and lighting; where as in the urban areas of developing countries where alternative energy exist, fuelwood still remains the main source of domestic energy [3–6]. Africa is rated the top highest fuelwood consumer in the world energy projections for the years 2010, 2020 and 2030 [7]. Africa's fuelwood consumption is expected to stand at 544.8 million m^3 and 46.1 million tons for firewood and charcoal respectively by 2030 [7]. Widespread use of fuelwood in the developing world and Africa in particular has been linked to woodland degradation, and poverty [8]. Fuelwood collection and use is on the increase in both the rural and urban areas with population growth and poverty as the most important contributory factors especially in Africa [9]. With the current 2.3% rate of global population growth [10], fuelwood use is expected to increase due to continuous dependence on it as the main household energy

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source. The current situation poses considerable challenge to the forests and woodland resources [11, 12].

This means that every effort must be made to ensure sustainable fuelwood collection by ensuring sustainable woodland management if the above targets must be met. The demand for fuelwood and its social consequences may worsen for the urban poor of developing countries particularly at this time that prices of petroleum products are on the increase. Fuelwood use is on the increase and concerted effort towards sustainable woodland management is crucial for sustainable fuelwood collection [13]. Notwithstanding the important role fuelwood plays globally and the growing interest in fuelwood use, its collection particularly for commercial purposes is widely criticised for its contribution to woodlands and forests degradation [4, 14, 15, 16, 7]. By their objective, commercial fuelwood collectors are those who harvest fuelwood for sale. By their nature they fell both live and dead trees preferably species with high calorific value. They also harvest both primary and secondary preferred tree species [17] depending on the kind of tools collectors use; chainsaw users prefer primary tree species which have high wood density and high market value while cutlass or axe users harvest secondary tree species because they are easy to cut though their wood densities are low. They also rent woodlands from the chiefs and landlords in countries such as Ghana. Poor woodland access triggers environmental degradation which in turn aggravates poverty and further worsens the situation of degradation [16]. The effects of fuelwood collection on woodlands and forest can be reduced through good management practices [18]. Understanding factors that affect fuelwood collection can improve management of woodlands in order to minimize the negative effects associated with commercial fuelwood collection and the consequences on human well-being.

Ghana depends heavily on fuelwood especially for her domestic energy needs. The pattern of fuelwood consumption in Ghana is not different from what is observed in other developing countries. Consumption is on the increase while the quantity and quality of woodlands are decreasing. Fuelwood constitutes about 70% of energy consumed in Ghana [11, 19]. Ghana is one of the countries with high per capita fuelwood demand in West Africa and among the top two in charcoal consumption, signifying the important role fuelwood plays in Ghana's socio-economic development [20, 21]. It is therefore not surprising when the Strategic National Energy Plan (SNEP) 2006-2020 projects a rise in fuelwood consumption from 14 million ton in 2000 to between 38 - 48 million ton by 2012, and 54 - 66 million ton by 2020 [11]. In Ghana, much of the fuelwood that is consumed in the urban centres of the country is produced in areas such as Kintampo North District (KND) within the transitional zone, which is ecologically fragile. Despite the ecological sensitive nature of these areas, commercial fuelwood collection is one of the main sources of income for the rural poor who defy conservation practices to make ends meet on daily basis. Besides, the transition zone is noted for the heavy presence of migrant population most of whom are engaged in fuelwood collection for commercial purposes. The migrants do not have ownership rights to the woodlands and thus rent woodlands from the chiefs and individual family heads. The ownership of planted trees on rented lands is not clearly defined in Ghana and does not encourage people to plant trees on rented lands to supplement fuelwood collection from natural woodlands [22]. Also, Land based economic activities have expanded leading to accelerated woodland conversion to farms and settlements. Thus woodlands are under severe pressure raising questions about their sustainability and future fuelwood supply.

The threat to sustainable woodland management and for that matter sustainable fuelwood collection poses a great challenge to the traditional sub-energy management in Ghana; particularly to meet the energy needs of the country between 2012 and 2020 as projected in the Strategic National Energy Plan [11]. It is therefore imperative to analyse the factors affecting sustainable fuelwood collection in the major commercial fuelwood producing areas at KND. This will contribute to solutions to sustainable commercial fuelwood collection while minimising its impacts on other natural resources. It is against the above views that the research was set out to analyse factors affecting fuelwood collection.

[II] MATERIALS AND METHODS

2.1. Study area

KND is one of the major producers of fuelwood within the transition zone of Ghana. Livelihood activities of majority of the people are natural resources based such as farming and commercial fuelwood collection [23]. The fuelwood collection is either the primary source of incomes or a supplement to the mainstream agriculture.

The district has many rivers and undulating topography **[Figure–1]** which influence the choice of fuelwood collection areas. KND is located between latitudes 8°45'N and 7°45'N and Longitudes 1°20'W and 2°1'W. The district has a surface area of about 5,108km². It shares boundaries with five other districts: West Gonja to the North, Bole to the West, East Gonja to the North-East, Kintampo South to the South, and Pru to the South-East. Some of these districts are also known for commercial fuelwood collection. The district is strategically located at the centre of Ghana and serves as a transit point for migrants from the northern part of the country [24]. The elevation of the terrain ranges between 60 -150m above mean sea level. The fuelwood collection is either the primary source of incomes or a supplement to the mainstream agriculture. The Tamale–Techiman–Kumasi trunk road passes through the district which facilitates the transportation of fuelwood to commercial markets in Accra, Kumasi and other urban centres thus creating a ready market for fuelwood. This has therefore made fuelwood collection a brisk business in KND.

KND falls under the interior wooded savannah. However, due to its transitional nature, the area does not totally exhibit typical savannah conditions. The savannah is heavily wooded, though most of the trees are not as tall and gigantic as those in the moist deciduous forest. It is believed that the transitional zone was once forested and that the savannah conditions currently prevailing have been as a result of man's

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activities such as agriculture, logging, bush fire [25] and fuelwood harvesting. KND experiences the interior Savannah type of climate as it is within the transitional zone of Ghana with the mean annual rainfall which is between 1,400mm-1,800mm

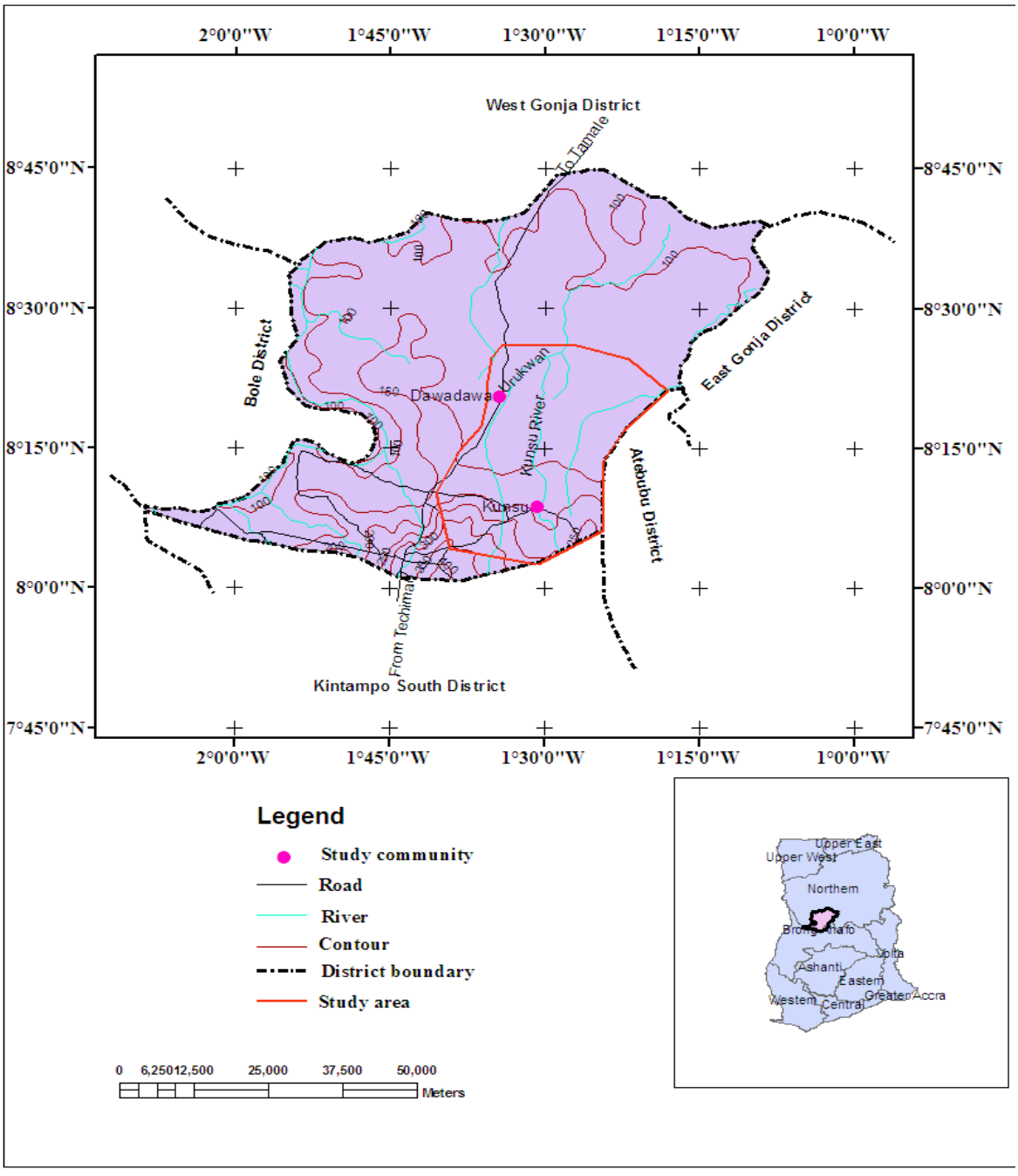

Fig: 1. Study Area

2.2. Materials and software

The Visible Near Infrared (VNIR) bands of Aster January 29th, 2007 image was used as the base map for the mapping of the fuelwood collection sites. Garmin 76S hand-held Global Positioning System (GPS) was used to pick sample locations of fuelwood collection sites in the field. A topographic map (1:50,000) was used as a guide during the reconnaissance survey and field visits. ERDAS Imagine 9.1 software was used to process the image; ArcGIS 9.2 software was used to overlay roads, rivers and contours with the image; and SPSS 15.0 software was used to process and analyse the socio-economic data. Microsoft Office Excel was also used to sort the GPS data for transformation into the Ghanaian local coordinate system.

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2.3. Sampling

Dawadawa and Kunsu are leading commercial fuelwood producing communities in the district [24] and were also purposively sampled because they were easily accessible by road at the time of the research. Each study community was stratified in order to have a representative sample. Major roads and paths were used as boundaries of the strata. Individual fuelwood collectors were purposively selected since commercial fuelwood collection was not carried out by everyone in the communities. The number of the respondents was determined by willingness of commercial fuelwood collectors to be interviewed because of the illegal nature of their activities and the time that was available for the research. In all, sixty commercial fuelwood collectors were interviewed in each community.

2.4. Focus group discussion and participatory mapping

A focus group of eight people was engaged in a discussion, centred on the identification of the major factors affecting commercial fuelwood collection in each community and mapping of the fuelwood collection sites. The fuelwood collection sites were mapped using participatory mapping whereby fuelwood collectors in each community identified and sketched areas where they collect wood. The mapping was done on geo-referenced Aster-2007 image overlaid with roads and boundaries of the study communities to improve the identification of the fuelwood collection sites. Participants mapped areas where preferred trees for fuelwood were completely harvested and areas that were being harvested at the time of the data collection. Participants referred to the distance from the settlements and rivers (Urukwan and Kunsu) to the collection areas. They also made reference to Tamale Techiman trunk road, Kintampo-Kunsu road, and neighbouring villages such as Attakura, and Kawumpe, Meawani and Adomano.

Two participants from the focus group were engaged in a field validation, which took the form of a mobile interview, through the fuelwood collection areas. The geographic locations of sample fuelwood collection sites were recorded using Garmin GPS 76S in order to validate the mapped areas.

2.6. Interviews and key informant discussions

Face-to-face interviews were conducted for fuelwood collectors in each community. Respondents were asked among other questions to rank the factors identified by the focus group discussions. Pair-wise ranking was used.

2.7. Data analysis

Weights were assigned to the ranks **[Table–1]** and used for statistical analysis in Statistical Package for Social Scientists (SPSS). The weights were assigned based on rank reversal. The most important factor by rank was assigned the highest weight while the least important factor was assigned the least weight. The mean weights, standard deviation, minimum and maximum weights of each factor were computed. The final ranking was done based on mean weight. The standard deviation, minimum and maximum weights served as explanatory variables. The greater the mean weight of a factor, the more it affects fuelwood collection. The factor with the greatest mean weight was ranked 1; meaning the most important factor affecting fuelwood collection and the factor with the least mean weight was ranked 6 meaning the least important factor affecting fuelwood collection

The fuelwood collection site map was scanned and geo-referenced in ArcMap. The boundaries of the collection sites were digitized on-screen. The boundaries together with the sample points were overlaid on the image.

Table: 1. Ranks and weights of factors affecting fuelwood collection

Rank	Weight Assigned
1	6
2	5
3	4
4	3
5	2
6	1

[III] RESULTS

3.1. Demographic characteristics of respondents

The major age group involved in commercial fuelwood collection in the communities is 30-39 years and constituted 50% and 43% of the respondents in Dawadawa and Kunsu respectively **[Figure–2]**. It is also evident from Fig. 2 that a number of respondents are of ages between 40 and 49 in both communities also engaged in commercial fuelwood collection. There were no collectors below 20 years.

The major source of income of commercial fuelwood collectors is fuelwood sale, 95% in Dawadawa and 92% in Kunsu **[Figure–3]**. Farming is a major source of income to 5% and 8% of the respondents in Dawadawa and Kunsu respectively, with fuelwood sale as a supplementary source. In Dawadawa, 70% of the respondents either had little or no formal education as compared to 80% in Kunsu. The rest of the respondents in both communities had either primary or Junior Secondary/Middle school education **[Figure–4]**.

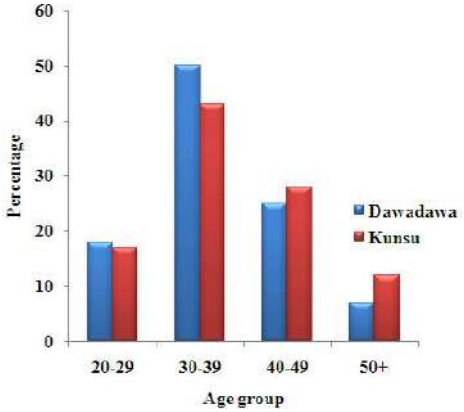

Fig: 2. Age distribution of FW collectors

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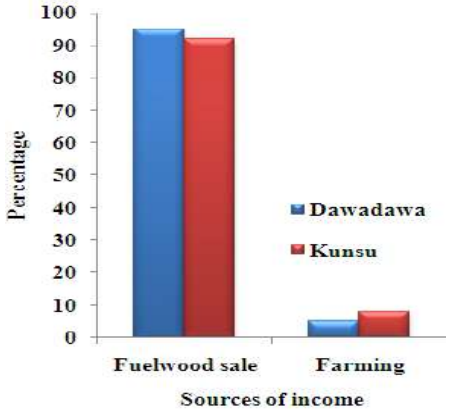

Fig: 3. Sources of income of FW collectors

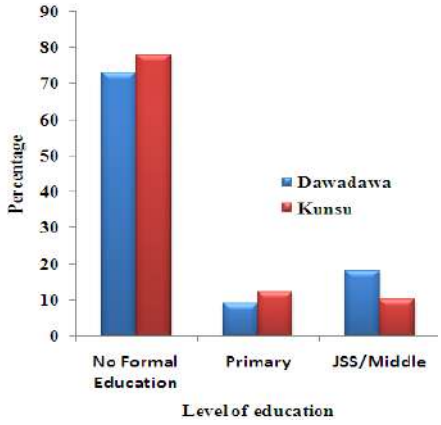

Fig: 4. Educational level FW collectors

3.2. Commercial fuelwood collection sites

Commercial fuelwood collection sites between the period 2000 and 2007 were studied **[Figure–5; Plate–1, Plate–2, Plate–3 & Plate–4]**. In the case of Dawadawa, collection site A was exhausted of harvestable preferred tree species **[Plate–1]**, site B **[Plate–2]** which is about 10km from the settlement still had few harvestable species and site C **[Plates–3 & 4]** which is about 20km **[Figure–6]** had a high density of harvestable preferred species. Site A and B **[Figure–5]** were dominated by *Afromosia spp*., *Nuclea latifolia*, *Ceiba pentandra*, *Adansonia digitata*, *Mitrogyna spp*., *Danialla olivery*, etc., which are not preferred species for commercial fuelwood **[Plates–1 and 2]**. Though, site A was exhausted of harvestable preferred species for commercial purposes, some domestic fuelwood collectors were found harvesting wood there **[Plate–1]**.

In Kunsu, respondents did not separate areas exhausted of harvestable preferred tree species from areas still dominated by harvestable preferred tree species since such areas do not exist in isolation as in the case of Dawadawa. Commercial fuelwood collectors collected fuelwood within 10km from the settlements **[Figure–6]**. Preferred tree species for commercial fuelwood collection in the study communities include *Lophira lanceolata, Pseudocedrela kotchyi, Albizia coriaria, Vitellaris paradoxa, Pterocarpus erinaceus, Anogeissus leiocarpus and Erthrophyleum guineensis.*

Plate: 1. **FW collection site– A**
Plate: 2. **FW collection site– B**
Plate: 3. **FW collection site–C**
Plate: 4. **FW collection site–C**

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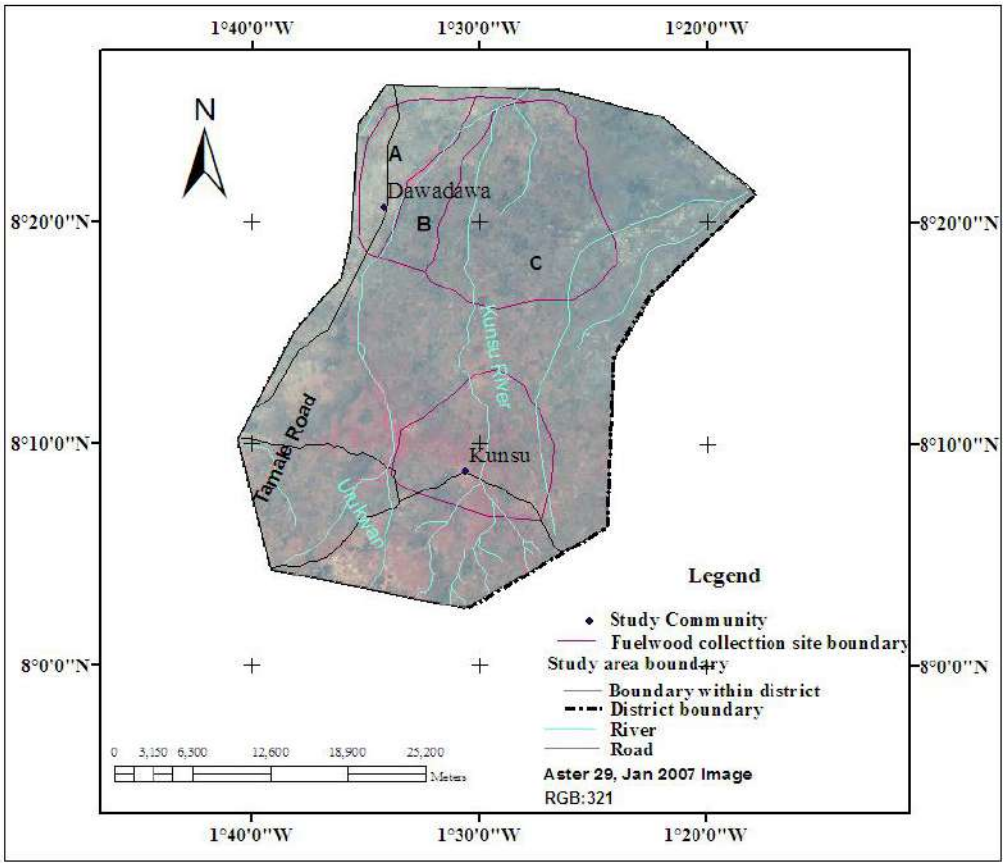

Fig: 5. Fuelwood collection site map

3.3. Factors affecting fuelwood collection

With the exception of poor roads, topography, slash and burn farming, and land tenure, the rest of the factors affecting commercial fuelwood collection are the same for the two communities **[Table–2]**. Land tenure and poor roads were not listed in Dawadawa as topography and slash and burn farming practice were not also listed in Kunsu.

Low producer price of fuelwood and poverty were ranked first and second respectively in Dawadawa **[Table–3]**. The mean weight of low producer price is marginally higher than that of poverty by 0.24 while the standard deviation is lower by 0.233. Bushfire is ranked least with a mean weight of 2.22 and standard deviation of 0.948. Slash and burn farming practices with the largest standard deviation of 1.203 is ranked fifth.

Table: 2. Factors affecting commercial fuelwood collection

Factor	Community	Effect of factor on fuelwood collection
Low producer price for fuelwood	Dawadawa and Kunsu	It leads to overexploitation of the woodlands.
Poverty	Dawadawa and Kunsu	It leads to over exploitation of woodlands.
Topography	Dawadawa	Increase cost of fuelwood collection during rainy season.
Rivers	Dawadawa and Kunsu	Reduce access to collection areas, increase cost of fuelwood collection during rainy season
Slash and burn farming	Dawadawa	Impediment to sustainable fuelwood collection as it destroys the woody vegetation.
Frequent bushfire	Dawadawa and Kunsu	Impediment to sustainable fuelwood collection, a disincentive to establishment of fuelwood plantations
Poor Roads	Kunsu	Inaccessibility to fuelwood markets.
Land tenure	Kunsu	Impediment in terms of access to wood resource and planting of trees on rented lands and management of trees.

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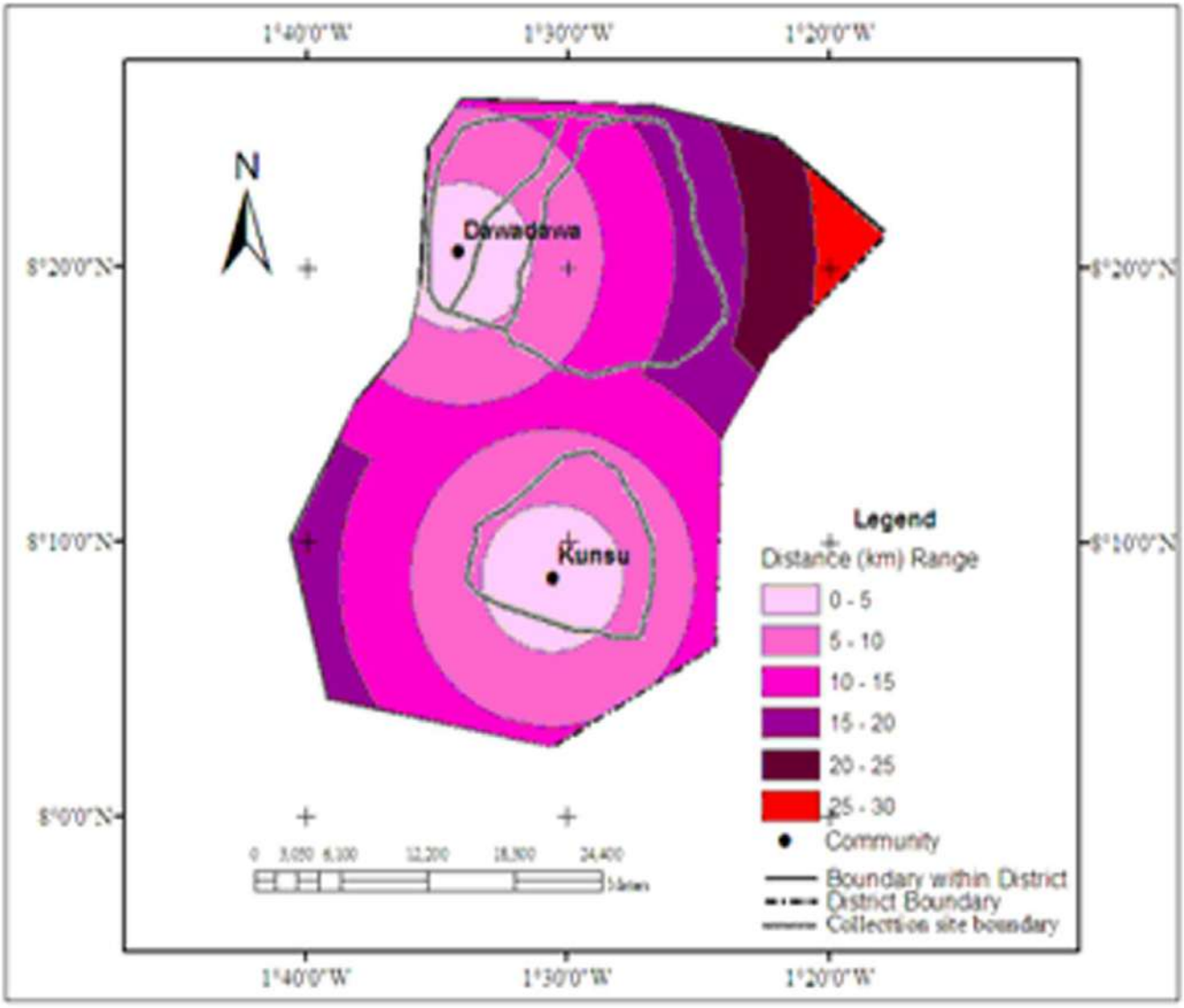

Fig: 6. Distance map showing distace from settlement to FW collection site

Table: 3. Statistics of factors identified in Dawadawa

Factors	Weights			Std. Dev	Rank
	Min	Max	Mean		
Low producer price of fuelwood	4	6	5.68	0.539	1
Poverty	4	6	5.44	0.772	2
Topography	3	6	4.51	0.917	3
Rivers	2	6	4.05	0.818	4
Slash and burn farming	2	6	4.00	1.203	5
Bushfire	1	4	2.22	0.948	6

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Land tenure was ranked as the most important factor affecting commercial fuelwood collection with the highest mean weight of 5.75 and a standard deviation of 0.439 in Kunsu [Table–4]. Bushfire was ranked the least factor with a mean weight of 1.42 and standard deviation of 0.675.

Table: 4. Statistics of factors identified in Kunsu

Factors	Weights			Std. Dev	Rank
	Min	Max	Mean		
Land tenure	5	6	5.75	0.439	1
Poverty	4	6	5.54	0.625	2
Low producer price of fuelwood	3	6	4.51	0.917	3
Roads	2	6	3.27	0.827	4
Rivers	1	4	2.17	0.699	5
Bushfire	1	3	1.42	0.675	6

[IV] DISCUSSION

4.1. Commercial fuelwood collection sites

Fuelwood is collected from woodlands in Dawadawa which are as far as 20 km from settlements. Collections sites in Dawadawa extend over large tracks of woodlands with current collection sites concentrated in the eastern part where preferred species are still abundant. Twenty (20) kilometre from settlement to collection sites in Dawadawa is considered a long distance [26]. It is a source of concern not only to the collectors but also management of the traditional energy sub-sector in Ghana since long distance from settlements to collection sites is a yardstick for fuelwood scarcity [26, 27]. Also some fuelwood collection sites are less than 10 km to the district boundary of Attebubu District which has the possibility of initiating conflict as collectors get closer to the district boundary.

4.2. Factors affecting fuelwood collection

Bush fire, and slash and burn farming practices were ranked low though these factors are noted for their devastating effects on woodlands in the study area. It is attributable to the fact that some fuelwood collectors were also farmers and did not agree that slash and burn farming destroys woodlands. This assertion is affirmed by the fact that, slash and burn farming has the highest standard deviation of 1.203 signifying a divided opinion in the ranking. Slash and burn farming was not identified in Kunsu though the practice goes on there and is because wood resource is abundant in Kunsu such that commercial fuelwood collectors do not realise its effects on sustainable fuelwood collection.

4.2.1. Land tenure system

Land tenure system was ranked first with the least standard deviation of 0.439 signifying agreement in the opinions of the respondents that land tenure system is really the most dominant factor affecting commercial fuelwood collection in Kunsu. Contrary to what was expected, it was not listed among the factors affecting commercial fuelwood collection in Dawadawa. This is ascribed to the perspective from which fuelwood collectors view land tenure in relation to woodland use and the land tenure system practised in each community. In terms of access to woodland, land tenure poses little difficulty to commercial fuelwood collectors once they are willing to pay for the woodland. In terms of sustainable fuelwood collection, it poses serious difficulties because land tenure system in Dawadawa does not ensure sustainable tree harvesting, such as enforcement of restrictions on sizes of trees to be harvested. The danger of such tenure system is that it leads to overexploitation of woodlands for commercial fuelwood collection as in Dawadawa. In Dawadawa, woodland is entrusted to the chief and he enjoys direct monetary benefits from renting the woodlands to commercial fuelwood collectors. Access to woodland is more or less open to them once they agree to pay the required tribute to the chief as rent. Thus it is expected that the chief takes responsibility of ensuring sustainability of the woodland such as restrictions on the type and size of trees to harvest [28]. Unfortunately, commercial fuelwood collectors do not have any motivation for sustainable harvesting because they rent the woodlands and this attitude begs enforcement of restriction on sizes of trees harvested for commercial fuelwood [29]. Land tenure system makes access to woodlands easy for commercial fuelwood collectors in Dawadawa and this explains why land tenure was not mentioned as a factor affecting commercial fuelwood collection. In Kunsu, woodland is entrusted to households who enjoy direct monetary benefits from renting woodlands to commercial fuelwood collectors. It is easy to enforce restrictions on access to woodlands because many people have interest to protect woodlands since they enjoy direct benefits from woodlands. Commercial fuelwood collectors are restricted to the number and size of tree species to harvest. These restrictions make access to woodland difficult for the migrant landless commercial fuelwood collectors and accounts for the ranking of land tenure system as the most important factor in Kunsu.

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The land tenure system practised in Kunsu ensures sustainability of woodlands whereas that of Dawadawa does not. It is therefore clear that land tenure is an important factor through which people gain access to land and natural resources. Though it poses some level of difficulties in accessing wood resource in Kunsu and elsewhere [30], it ensures sustainable harvesting of wood.

4.2.2. Poverty

Most of the fuelwood collectors are poor and find it difficult to make ends meet on daily basis. The urban wholesalers capitalise on it and offer low producer-price for fuelwood. The poverty situation of the collectors makes it difficult for them to reject such undeserving prices offered by the urban wholesalers. The implication is that fuelwood collectors continue to work for hand-to-mouth and are not encouraged to establish private fuelwood plantations. Consequently fuelwood collectors continuously depend on the natural woodland and greatly contributing to the degradation of woodland as demonstrated in Dawadawa.

4.2.3. Low producer price of fuelwood

The ranking of low producer-price of fuelwood first and third in Dawadawa and Kunsu respectively shows the low economic benefits derived from commercial fuelwood collection. It indicates that fuelwood collection is not yielding the expected economic benefit. This has a negative implication on the sustainability of the woodlands in terms of protecting the woodlands against annual bush fires.

In Dawadawa for instance, for renting a chainsaw per operation, the fuelwood collector pays US$35.00, one-third of this amount is meant for fuel to operate the chainsaw, another one-third for maintenance of the chainsaw and the rest is meant for the labour of the chainsaw operator. The activities covered by the US$35.00 constitute a set of fuelwood harvesting operation. One harvesting operation yields an average of 100 bags of fuelwood depending on the type of three species and the expertise of the fuelwood collector. For every ten bags of fuelwood produced, one bag (US$3.00) is given to the chief as rent for the woodland. Therefore, for one harvesting operation (100 bags of fuelwood), the total rent is US$30.00 To transport a bag of fuelwood from the collection point to the sale point, it costs on the average US$1.00 depending on the topography of the terrain, the season of the year and the distance between the collection and the sale points. The total cost of transport per a harvesting operation becomes US$100.00. The time and energy spent by the fuelwood collector is estimated to worth US$ 1.00 for every bag of fuelwood produced. For the 100 bags of fuelwood produced per operation, the total time and energy spent is worth US$ 100.00. This brings the total cost of production to US$265.00 (US$35.00 + US$30.00 + US$100.00 + US$100.00) for one harvesting operation. If a bag of fuelwood is sold for US$3.00, then the total revenue generated for harvesting operation amounts to US$ 300.00 resulting in a profit of US$35.00 for a harvesting operation. This means that the producer profit per bag is US$0.35. A similar analysis applies to Kunsu. These expenses make producer-price of US$3.00 in Dawadawa and US$2.00 in Kunsu for a 46 kg bag of fuelwood worthless. These prices are far below the average prices of $8.00, $9.00 and $10.00 for the same quantity of fuelwood in the urban markets in Tamale, Kumasi, and Accra respectively. Evidence from field observation shows that the low producer price is due to lack of policy to regulate the prices of fuelwood nationwide; partly because fuelwood collectors in the study area do not have any recognised group to press home their needs. The fact that commercial fuelwood collectors do not have recognised groups has negative implications on the enforcement of conservation regulations because it is more difficult to monitor an individual than a group.

4.2.4. Topography and poor roads

Among the biophysical factors, topography was ranked the most important factor for commercial fuelwood collection in Dawadawa because of the flat nature of the terrain (0 – 2% slope). Topography was not mentioned in Kunsu because there were alternative collection sites in times of floods. Flooding affects fuelwood collection since it denies fuelwood collector's access to collection sites.

The quality, density and length of roads to market centres are critical to the profitability of commercial fuelwood collection. The roads facilitate fuelwood transportation to market centres in the urban areas. It is ranked fourth among the factors affecting fuelwood collection in Kunsu. This is due to the deplorable state of the main Kunsu-Kintampo road which limits access to the nearest urban centre (Kintampo). The effect of the poor state of the road is translated into low producer-price for fuelwood in Kunsu; since it becomes difficult for vehicles to convey fuelwood from Kunsu to Kintampo for onward transportation to Kumasi and Accra. Poor roads were not identified in Dawadawa because the community is linked to the market centres by the Tamale–Techiman highway which facilitates the transportation of fuelwood.

[V] CONCLUSION

Fuelwood collection is a major source of livelihood for commercial fuelwood collectors at KND. Land tenure can be an incentive or a disincentive in woodland management for fuelwood collection.Woodlands in Dawadawa are under severe pressure to support livelihoods of commercial fuelwood collectors since the land tenure system does not ensure sustainable harvesting of wood. Woodlands are managed sustainably in Kunsu compared to Dawadawa due to effective tenure system that is practised in Kunsu. The land tenure system and low producer price of fuelwood are major disincentives to the establishment of private plantations to supplement diminishing natural woodlands in the study area.

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Finances so far are borne by authors and subsequent expenses will still be borne by authors.

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RESEARCH: BIOREMEDIATION

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PHYSICO CHEMICAL ANALYSIS OF TEXTILE EFFLUENT AND DECOLORIZATION OF TEXTILE AZO DYE BY *BACILLUS ENDOPHYTICUS STRAIN* VITABR13

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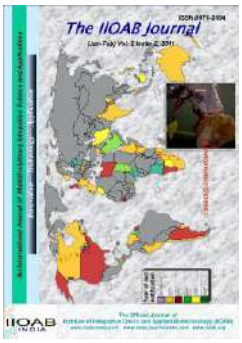
ABSTRACT



The physicochemical characterization of the textile industry effluent collected from Coimbatore in Tamil Nadu,India was been carried out and the results showed high rates of temperature (40°C), pH (7.51) and Electrical Conductivity (9565 µmhos/cm), Biological Oxygen Demand (275 mgl^{-1}), Chemical Oxygen Demand (789 mgl^{-1}), Total Suspended Solids (1750 mgl^{-1}), Total Dissolved Solids (5875mg l^{-1}), heavy metal ions, Total hardness (Ca^{2+}, Mg^{2+}, Cl⁻ & SO_4^{2-}) and colour over the prescribed fresh water limits. A potential bacterial strain(VITABR13) was isolated and selected from the textile effluent on the basis of rapid azo dye Acid Red 128(100mgl^{-1})decolorization and later identified as belonging to genus Bacillus based on Phenotypic characterization and phylogenetic analysis of the 16s rRNA gene sequence. Effects of physicochemical parameters (pH, Temperature, Carbon and Nitrogen sources) on the Acid Red 128 decolorization by the selected bacterium were studied. Decolorization was effective at pH 8, 35°C with starch and peptone as carbon and nitrogen sources and in static conditions. This decolorization potential increased the applicability of this microorganism for the dye removal.

.

Keywords: *textile Industry effluent; Bacillus endophyticus VITABR13; acid red 128; decolorization*

[I] INTRODUCTION

Water pollution control is at present one of the major areas of scientific activity. Textile industries are large industrial consumers of waters as well as producers of wastewaters. With the increased demand for textile products, the textile industry and its wastewaters have been increasing proportionally, making it one of the main sources of severe pollution problems worldwide [1,2]. The diversity in composition of chemical reagents used in textile industries contributes to much of the water pollution. The reagents range from inorganic compounds to polymers and organic products [3]. Waste water generated by different production steps of a textile mill have high pH, temperature, detergents, oil, suspended and dissolved solids, dispersants, leveling agents, toxic and non-biodegradable matter, color and alkalinity. [4]. Important pollutants in textile effluent are mainly recalcitrant organics, color, toxicants and surfactants, chlorinated compounds (AOX). The textile wastewaters are characterized by extreme fluctuations in many parameters such as chemical oxygen demand (COD), biochemical oxygen demand (BOD), pH, colour and salinity [5].The process of adding colour to the fibres is known as dyeing which normally requires large volumes of water not only in the dye bath, but also during the rinsing step? The process of dyeing involves the use of different chemicals like salts, metals, surfactants, organic processing assistants, sulphide and formaldehyde. There are more than 8,000 chemical products associated with the dyeing process and over 100,000 commercially available dyes exist with over 7×10^5 metric tons of dyestuff produced annually [6].

Approximately a half of all known dyes are azo dyes, making them the largest group of synthetic colorants. Azo dyes consist of a diazotized amine coupled to an amine or phenol. Atleast 3000 different varieties of azo dyes are extensively used in the textile, paper, food, cosmetics and pharmaceutical industries [7]. Azo dyes are characterized by the presence of one or more azo groups (R_1-N=N-R_2) which are responsible for their colorations and when such a bond is broken (degraded) the compound loses its color [8].A related topic having received considerable interest is the presence of color in effluents associated with production of dyes. Color in the effluent is one of the most obvious indicators of water pollution and the discharge of highly colored synthetic dye effluents is aesthetically displeasing and can damage the receiving water

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body by impeding penetration of light [9].Dyes are recalcitrant molecules which are difficult to degrade biologically. Some of azo dyes are either toxic or mutagenic and carcinogenic [10].Azo dyes are designed to resist chemical and microbial attacks and to be stable in light and during washing [11].Treatment of dye waste water involves chemical/physical methods, among which are coagulation, precipitation, adsorption by activated carbon, oxidation by ozone, ionizing radiation and ultrafiltration.These physico- chemical methods are generally costly, produce wastes which are difficult to dispose of, are less efficient and of limited applicability [12].As a viable alternative, biological processes have received increasing interest owing to their cost effectiveness, ability to produce less sludge, and environmental friendliness [13].Microorganisms capable of degrading azo dyes include *proteus spp* [14],*Enterococcus spp* [15], *Streptococcus spp*[16],*Bacillus cereus* [17], *Streptomyces spp* [18], and the white rot fungus *Chanerochaetes chrysosporium* [19].Decolorization of azo dyes normally begins with initial reduction or cleavage of azo bond anearobically, which results into colorless compounds. This is followed by complete degradation of aromatic amines strictly under aerobic conditions [20].The effectiveness of microbial decolorization depends on the survival, adaptability, and activity of the microorganism [21].Azo dyes generally resist aerobic microbial degradation, only organisms with specialized azo dye reducing enzymes were found to degrade azo dyes under fully aerobic conditions [22]. Although there are many studies on microbial decolorization of azo dyes by various microbial species, it is necessary that new experiments be conducted for finding new resources and microorganisms with suitable biological properties for decolorization.These new microorganisms which have capability to grow in polluted environment, minimal nutritional requirements and rapid growth can be used to achieve good results in decolorization experiments [23]. Hence, the Objective of our present study was: 1) to characterize the textile effluent for its physico-chemical parameters 2) To assess the potential of *Bacillus endophyticus* VITABR13 strain isolated from textile effluent to decolorize an azo dye (Acid Red 128) and to optimize the various physico chemical parameters such as pH, Temperature, Carbon and Nitrogen sources for efficient dye decolorization.

[II] MATERIALS AND METHODS

2.1. Sampling and analysis of effluent

Coimbatore is one of the most industrialized cities in Tamil Nadu, India. It is known as the textile capital of South India or the Manchester of the South India (Lat.11° 00'N, Long. 77° 00' E) and was chosen for effluent sample collection. The Effluent sample was collected from the middle point of the area. Standard procedures (Spot and Grab) were followed during sampling. The Temperature and pH were determined at the sampling site. The pH was determined by using pH meter (Hanna digital pH meter, model-671-p) and temperature with laboratory thermometer. The sample was transported to laboratory at 4ºC as in accordance with the standard methods [24].

The physicochemical parameters such as (Colour, Electrical Conductivity (EC), Biological Oxidation Demand (BOD) Chemical Oxygen Demand (COD), Total Suspended Solids (TSS), Total Dissolved Solids (TDS), heavy metal ions) were determined as soon as the sample was brought to the laboratory. Sample colour was analysed by U-3010 spectrophotometer (Hitachi, Japan) while Electrical Conductivity(EC) was determined by conductivity meter (Jenvway EC meter, model-4070).BOD was determined by employing evaporation method by DO meter while COD was measured by COD instrument directly. Chloride and Sulphate contents were assessed by titrimetric and turbidity method, respectively [25]. The phenolic compounds were determined by photometric method. Different metal ions present in the effluent sample were determined by Atomic Absorption Spectrophotometer (AAS) as per the standard methods.

2.2. Chemicals

The textile dye Acid Red 128(λmax. 523nm) was obtained from a small Dyeing Industry in Coimbatore, Tamil Nadu. Nutrient broth (gL^{-1}Peptone-5, Meat extract-1, Yeast extract-2, Nacl-5, pH-7) A stock solution of the dye (1000mg L^{-1}) was prepared in de-ionized water and used for all studies.

2.3. Isolation, screening and identification of dye decolorizing bacteria from effluent

The Textile Effluent was collected in sterile collection tubes from the sludge and wastewater of the ditches at industrial site located in Coimbatore. The sample collected from the textile mill was screened for azo dye (Acid Red 128) decolorizing bacterial strains by inoculating 10 ml. of sludge solution into 250ml.Erlenmeyer flask containing 100ml. Nutrient broth (gL^{-1}Peptone-5, Meat extract-1, Yeast extract-2, Nacl-5,pH-7). The flasks were incubated at 35°C under shaking conditions (130rpm).After 48h of incubation, 1.0ml. of the culture broth was appropriately diluted and plated on Nutrient Agar (gL^{-1} Peptone-5, Meat extract-1, Yeast extract-2, Nacl-5, Agar-15, pH – 7.0) containing 20mg L^{-1} Acid Red 128.The Morphologically distinct bacterial isolates showing clear zones around their colonies due to decolorization of dye were selected for further studies. The pure culture stocks of these isolates were stored at 4°C on Nutrient Agar slopes containing 1000mg L^{-1} of Acid Red 128.These isolates were screened for their ability to decolorize Acid Red 128 in liquid culture.

The Screening process in liquid media was carried out by inoculating a loop full of cultures exhibiting clear zones into Nutrient broth containing Acid Red 128 under static conditions. After 24h of incubation, 1ml. of cell suspension was transferred to fresh nutrient broth containing Acid Red 128 to screen the strains with color removing ability. The Screening procedure in liquid medium was continued until complete decolorization of broth. A small amount of decolorized broth was transferred to nutrient agar plates containing Acid Red 128 (50 mg L^{-1}).The bacterial isolate which tolerated higher concentration of the Azo dye was isolated by streak plate method. The Azo dye decolorizing bacteria was identified from several aspects including morphology characters, biochemical tests, and sequence analysis of nearly complete 16srRNA gene.

The identification and characterization of the potential isolate was done by gram staining, motility, presence of spores, spore position and spore

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morphology and by following biochemical tests as described in Bergey's manual of determinative bacteriology (Indole, Methyl Red,Voges-Proskauer test, Citrate, Catalase, Oxidase, Nitrate Reduction test, Hydrolysis of Casein, Starch, Urea and Gelatin).Assimilation of various sugars such as D-glucose, D-fructose, galactose, mannitol and D-maltose as sole carbon source was determined by inoculating the isolate into carbohydrate broth supplemented with respective carbon source. After inoculation the tubes were incubated at 37ºC for 24 - 48h.The Molecular identification of the potential strain was carried out by amplifying the 16srRNA gene as described previously [26].The PCR product was purified using the QIA quick PCR purification kit (Qiagen).Sequencing was performed by using an ABI PRISM model 3700 automatic DNA sequencer and the big dye terminator cycle sequencing kit (both from Applied Biosystems).The almost complete 16srRNA gene sequence (1565 bp) was aligned with closely related sequences retrieved from EMBL by using CLUSTAL W [27]. Pairwise evolutionary distances were computed by using MEGA-4 software. Phylogenetic tree was constructed using NEIGHBOR, UPGMA, KITSCH, FITCH and DNAPARS of the PHYLIP package [28].The stability among the clades of a phylogenetic tree was assessed by taking 1000 replicates of the dataset with a cut off value of 50.

2.4. Decolorization assay

The decolorizing activity was expressed in terms of the percentage decolorization by the modified method described previously [29]. The Decolorization process was carried out using shaking culture and static culture by inoculating 1ml. of precultured (O.D 0.8-1) *B.endophyticus* VITABR13 into 100ml. of sterilized Nutrient broth in 250ml.Erlenmeyer flask and incubated on rotary shaker (130rpm) at 35ºC for 24h [30]. Filter sterilized (0.22 µm) Acid Red 128 (100 mgL^{-1}) was added to the culture and incubated in shaking conditions at 130rpm and in static conditions at room temperature for decolorization to occur. At regular intervals, 4ml.sample was withdrawn aseptically and centrifuged at 10,000 rpm for 15min.The cell free supernatant was used to determine the percentage decolorization of Acid Red 128.Decolorization of dye was determined by monitoring the decrease in absorbance at the maximum wavelength of Acid Red 128(λmax. 523nm) by using a UV-Visible spectrophotometer (UV-1700 pharmaspec, shimadzu). The uninoculated dye Medium supplemented with respective dye was used as blank [31]. Decolorization activity (%) was calculated by the following formula and all assays were done in triplicate:

$$\% \text{ decolorization} = \left(\frac{\text{Initial Absorbance} - \text{Final Absorbance}}{\text{Initial Absorbance}} \right) \times 100$$

2.5. Decolorization of acid red 128 under different culture conditions

The decolorization efficiency of *Bacillus endophyticus* VITABR13 strain was compared over a wide range of pH (5-9) by adjusting the pH with hydrochloric acid or sodium hydroxide.Decolorization at different Temperatures (RT, 35°C, 37°C, 40°C, 45°C, 50°C) was carried out by adjusting the pH to 8.Varying Carbon sources 1% each (dulcitol, starch, maltose, sucrose, dextrose, mannitol, d-xylose, lactose, mannose)and Nitrogen sources 1% each (urea, potassium nitrate, sodium nitrate, malt extract, ammonium sulphate, ammonium nitrate, ammonium chloride, peptone) were used to check the decolorizing potential of the strain. All the flasks were incubated in static conditions at pH 8 and at 35°C.

2.6. Statistical analysis

Data was statistically defined by one-way ANOVA using Microsoft excel. Results in each experiment were interpreted depending upon probabilities. Probability (p-value) was less than 0.05 which was found to be significant.

[III] RESULTS

3.1. Physico chemical characterization of textile effluent

The effluent sample collected from a small scale Textile Dyeing Industry in Coimbatore, Tamil Nadu, India, was black in colour, with pungent smell and pH of slightly above neutral level and was within the permissible limits. The temperature of the effluent was high. Electrical Conductivity (EC) of the effluent was low. Total Suspended Solids (TSS) and Total Dissolved Solids (TDS) in the textile effluent were very high. The solids present in ground water, besides effecting the growth of the plants directly, also affect the soil structure, permeability and aeration, indirectly effecting the plant growth. The Chemical Oxygen Demand (COD) and Biological Oxygen Demand (BOD) values were within the permissible limits in the effluent sample. The results of heavy metals analyzed in the textile effluent such as $Cu2^{+}$, Cd^{2+}, Zn^{2+}, Fe^{2+}, Cr^{3+}, Mn^{2+}, Ni^{2+}, Pb^{2+} had showed their amounts to be considerably high. Among the metals the level of Iron was the highest. The levels of Calcium (Ca^{2+}) and Magnesium (Mg^{2+}) metal cations in the effluent whose combined effect leads to the total hardness of water was also found to be very high. The sulphate ion (SO_4^{2-}) content was found to be within permissible limits but the chloride (Cl^{-}) content was found to be remarkably high which is an index of surface pollution level. The high salt concentration in ground water leads to formation of a saline soil and is a serious hazard to agriculture. The phenolic content was found to be greater than 0.1ppm which is though permissible but still toxic **[Table–1]**. Different bacterial strains isolated from the textile effluent were screened for their ability to decolorize the textile Azo dye (Acid Red 128) and the potential strains were characterized morphologically, biochemically and at molecular level for identification. The bacterial count (CFU/ml) was significantly high.

3.2. Isolation and identification

The study was started by screening for potential textile Azo dye decolorizing bacteria isolated from the textile industry effluent. Colonies surrounded by a nearly decolorized zone were isolated and then tested for dye removal capability using submerged culture. Strains isolated from the white colonies were inoculated in 100ml.of Nutrient broth in a 250ml.conical flask and incubated at 35°C under static conditions. One strain

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exhibiting highest decolorizing activity was chosen for further studies. The gram staining test showed the isolate to be non-motile, gram positive, spore forming, rod-shaped bacteria. The spore was terminally located and ellipsoidal in shape. Biochemical characterization of the isolate revealed it to be negative for Indole, Methyl Red test, Voges-Proskauer, Citrate, Catalase, oxidase test and Nitrate Reduction test. The isolate showed negative result for the hydrolysis of casein, gelatin, starch and urea.The strain utilized various sugars, D-Maltose, D-Glucose, D-Fructose, Mannitol and Galactose as sole carbon sources and was found to be positive **[Table–2]**. The potential strain was phylogenetically identified as *Bacillus endophyticus* VITABR13 on the basis of 16S rRNA gene sequence (1556bp).The sequence was compared with other sequences from GenBank database using BLAST (http://www.ncbi.nlm.nih.gov/BLAST/).The strain VITABR13 was closely related to *B.endophyticus* (2DT) AF295302 (98%) **[Figure–1]**. The sequence of the 16S rRNA gene of the strain VITABR13 is available under the GenBank accession number GU014293.

Table: 1. Physicochemical characterization of the textile effluent

S.No	Parameter	Units	Effluent
1.	Colour	-	Black
2.	Smell	-	Pungent
3.	Temperature	° C	40
4.	pH	-	7.51
5.	EC	µ mhos/cm	9565
6.	TSS	mg l^{-1}	1750
7.	TDS	mg l^{-1}	5875
8.	COD	mg l^{-1}	789
9.	BOD	mg l^{-1}	275
10.	Cu^{2+}	mg l^{-1}	3.62
11.	Cd^{2+}	mg l^{-1}	0.5
12.	Zn^{2+}	mg l^{-1}	1.0
13.	Fe^{2+}	mg l^{-1}	6.42
14.	Cr^{3+}	mg l^{-1}	1.5
15.	Mn^{2+}	mg l^{-1}	5.4
16.	Ni^{2+}	mg l^{-1}	0.4
17.	Pb^{2+}	mg l^{-1}	0.37
18.	Ca	mg l^{-1}	1500
19.	Mg	mg l^{-1}	889
20.	Cl^{-}	mg l^{-1}	2013
21.	SO_4^{2-}	mg l^{-1}	240
22.	Phenol	mg l^{-1}	0.143
23.	Bacterial Count	CFU/ml	11.6×10^5

3.3. Effect of pH and temperature on decolorization

The decolorization efficiency of *B.endophyticus* VITABR13 was compared across a range of pH (5-9).The maximum decolorization (90%) was recorded at pH 8. At neutral pH the strain exhibited percentage decolorization value of 77%.Where as it was 47% and 44% at pH 6 and 9.The percentage decolorization decreased markedly at pH 5 (8%) due to acidic conditions **[Figure–2]**. The optimum pH for growth and decolorization was found to be 8.The dye decolorization activity of the strain was found to decrease with increasing incubation temperature. Highest decolorization was achieved at

Table: 2. Morphological and biochemical characteristics of VITABR13.

Character	VITABR13
Gram staining	+
Morphology	**Rods**
Motility	-
Spore	+
Spore position	**Terminal**
Spore shape	**ellipsoidal**
Indole	-
Methyl Red	-
Voges-Proskauer	-
Citrate test	-
Catalase test	-
Nitrate Reduction test	-
Oxidase test	-
Hydrolysis of:	
Casein	-
Gelatin	-
Starch	-
Urea	-
Acid from:	
D-Maltose	+
Mannitol	+
D-Glucose	+
D-Fructose	+
Galactose	+

35°C (90%) and least percentage decolorization was at Room Temperature (RT) (27%).At 37°C there was 82% decolorization noted followed by 67%, 50% and 29% at 40°C, 45°C and 50°C respectively at the end of 24h incubation **[Figure–3]**. No specific decolorization was observed in shaking conditions (130rpm). (Data not included)

3.4. Effect of different carbon and nitrogen sources on acid red 128 decolorization

Results of Acid Red 128 decolorization by VITABR13 with different Carbon **[Figure–4]** and Nitrogen sources **[Figure–5]** are depicted. Dextrose resulted in better decolorization efficiency with 91% followed by starch (78%) and mannose (62%) at the end of 24h incubation period. The decolorization efficiency decreased with dulcitol (56%), mannitol (42%), lactose (37%), d-xylose (34%) and sucrose (28%).Least decolorization was observed with maltose (11%). Maximum decolorization with nitrogen sources was achieved with Peptone (87%) and least was with Malt extract (16%).Urea and Ammonium sulphate exhibited good decolorization with 77% and 61%.The decolorization efficiency decreased markedly with Ammonium nitrate (57%), Sodium nitrate (26%), Potassium nitrate (22%) and Ammonium chloride (21%).

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[IV] DISCUSSION

Industrial effluent is not stable and it varies often in a wide range depending upon the process practiced. South Asian countries are experiencing severe environmental problems due to rapid industrialization. This phenomenon is very common where the polluting industries like textile dyeing, leather tanning, paper and pulp processing, sugar manufacturing, etc.thrive as clusters. Among these the Textile industries are large industrial consumers of waters as well as producers of wastewater. The effluent discharged by this industry leads to serious pollution of groundwater and soils and ultimately affects the livelihood of the poor [32]. The physico-chemical characterization of the collected textile effluent sample from Coimbatore showed a high load of pollution indicators. Colour is contributed to a water body by the dissolved compounds (dyes and pigments). The effluent color was black due to mixture of various dyes and chemicals used in the dyeing process. [33]. The pH of the study sample was slightly alkaline when compared to the acidic pH of the dyeing effluent in a previous study [34]. The pH of the effluent alters the physico-chemical properties of water which in turn adversely affects aquatic life, plant and humans. The soil permeability gets affected resulting in polluting underground resources of water [35]. The temperature of the effluent was high in comparison with the temperature of another effluent in one study [36]. High temperature decreases the solubility of gases in water which is ultimately expressed as high BOD/COD. The values of BOD and COD were within the permissible limits in the present sample in comparison to the very high values of BOD and COD in one effluent study.

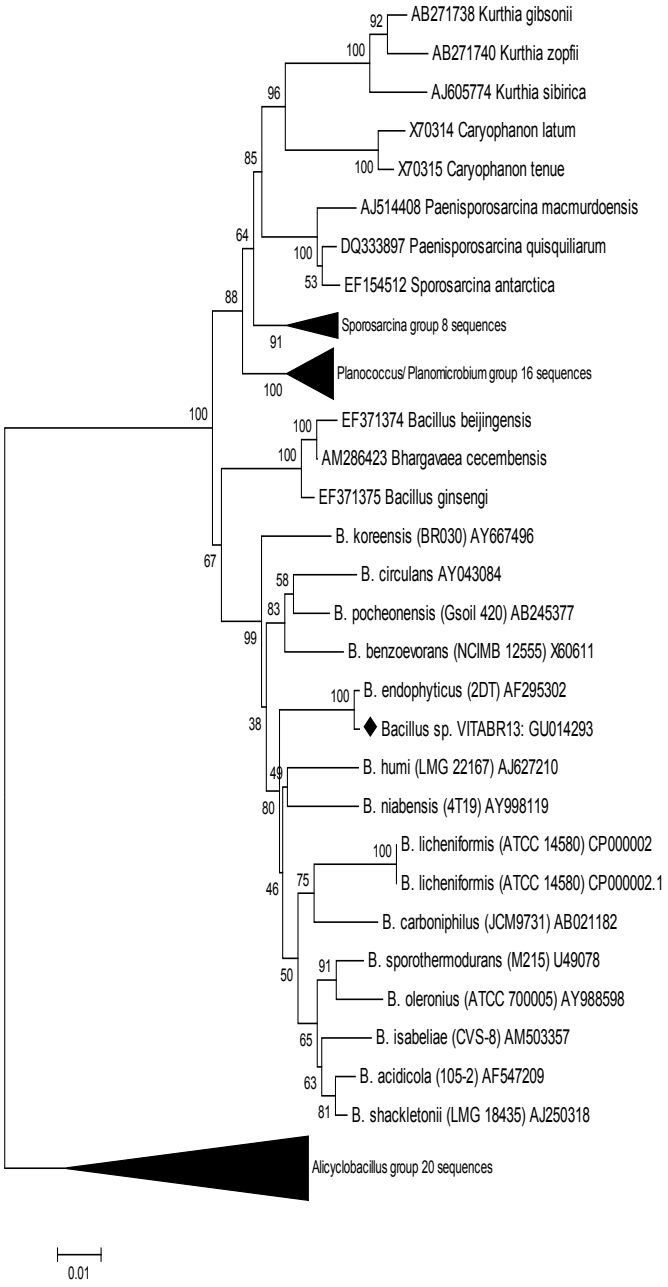

Fig: 1. Phylogenetic analysis of 16s rRNA sequence of Bacillus sp.VITABR13. The per cent numbers at the nodes indicate the levels of bootstrap support based on neighbor-joining analyses of 1,000 replicates.The scale bar (0.01) indicates the genetic distance.

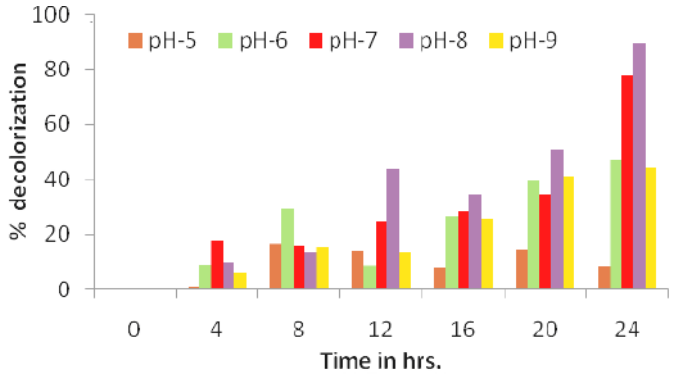

Fig.2 Acid Red 128 decolorization at different pH

The Electrical Conductivity of the sample was low. TDS and TSS values of effluent sample was high than the permissible limits but when compared to a textile effluent collected from a mill near Hisar (Haryana) was found to be low [37].Sediments rate is drastically increased because of High value of Total Dissolved Solids which reduces the light penetration into water and ultimately decrease the photosynthesis. The decrease in photosynthetic rate reduces the DO level of wastewater which results in decreased purification of wastewater by microorganisms [38].The current sample exhibited high values of heavy metals which was of the same order of magnitude reported in another effluent sample [39].The nutrients of the surrounding soils are depleted as a result of high value of heavy metals thereby affecting soil fertility.The Textile effluent had low values of sulphate and very high values of chloride content which are nearly equal to the value of one of the dyeing effluent sample from Faisalabad, Pakistan [40] .High chloride contents are harmful for agricultural crops if such wastes containing high chlorides are used for irrigation purposes. [41]. Majority of the

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textile effluent samples have permissible limits of sulphate ions. The effluent showed phenolic contents greater than 0.1ppm which is though permissible limit of the phenolic compounds still these compounds are very toxic to fish even at very low concentrations [42]. The bleaching and dyeing process are the main causes of pollutants which include caustic soda, hypochlorite and peroxides.

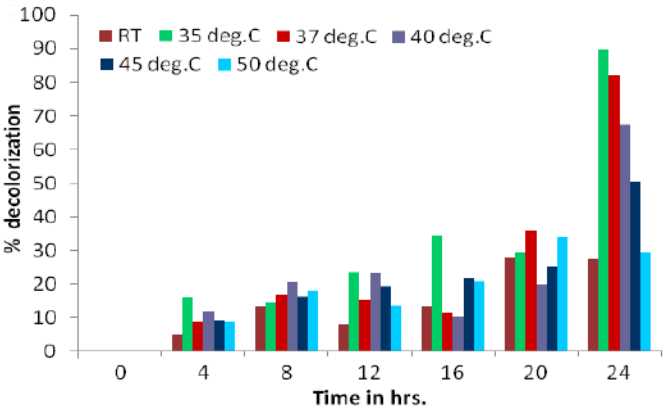

Fig: 3. Acid Red 128 decolorization at different Temperatures

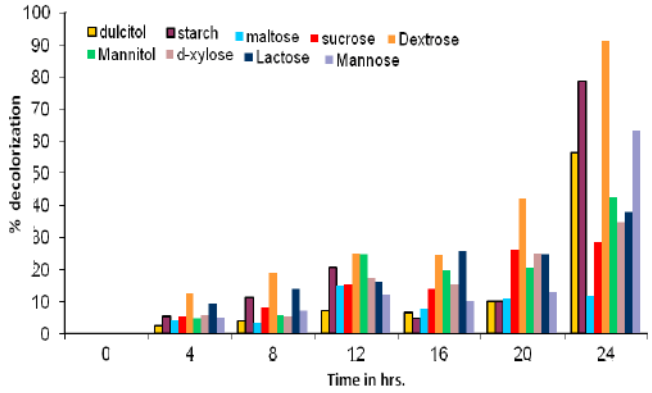

Fig: 4. Acid Red 128 decolorization in different C-sources

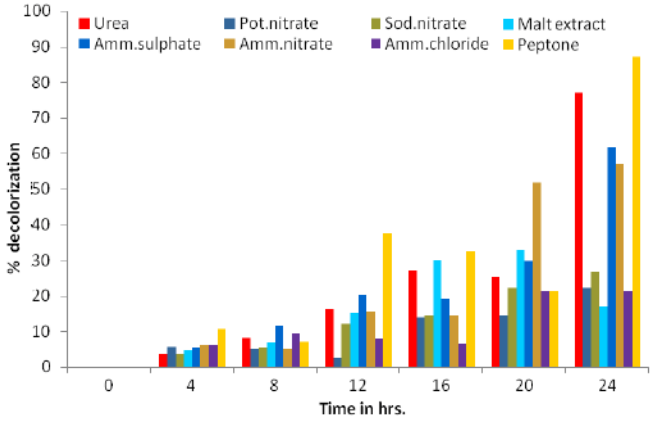

Fig: 5. Acid Red 128 decolorization in different N-sources

The isolation of different microorganisms from the effluent sample collected from the Textile Industry in Coimbatore indicates to natural adaptation of microorganisms to survive in the presence of toxic chemicals and dyes. Interest in the bioremediation of pollutants using bacteria has intensified in recent years, as many researches demonstrated the efficacy of bacterial bioremediation over fungal and Actinomycetes. Many bacteria capable of reducing Azo dyes reported were isolated from Textile effluent contaminated sites [43]. A strain of bacterium *Bacillus endophyticus* VITABR13 with strong decolorizing ability was isolated from Textile effluent to decolorize the textile Azo dye Acid Red 128(100 mgL^{-1}) within 24h in aerobic and static conditions. The reason for the decreased decolorization under shaking conditions could be competition of oxygen and dye compounds for the reduced electron carriers under aerobic conditions [44]. The percentage decolorization of Acid Red 128 by Bacillus *endophyticus* VITABR13 strain under static conditions was 90% within 24h of incubation which was equal to a similar study but with 35h of incubation period[45]. In another study conducted with *Pseudomonas putida, P.fluorescence, Bacillus cereus and Stentrophomonas acidaminiphila* to decolorize Acid Red 88 showed their efficiencies at 35%, 31%, 40% and 50% respectively [46]. Under aerobic conditions azo dyes are generally resistant to attack by bacteria [47]. The optimal pH for complete decolorization of Acid Red 128 was at 8 which is slightly in accordance with *Cosmarium sp.* Decolorizing malachite green at pH 9 [48] and *Klebsiella pneumonia* RS-13 which completely degraded Methyl Red in pH range of 6 to 8[49]. Optimal growth temperature of VITABR13 was found to be 35°C which is consistent with the highest decolorization temperature in our study. Maximum potential of *Pseudomonas sp.* to decolorize Malachite green, Fast green was noticed at 37°C [50].*Vibrio logei* and *Pseudomonas nitroreducens* showed the highest Methyl Red degradation activity at 30-35°C [51].Starch and Peptone were found to be most effective carbon and nitrogen sources for decolorization of Acid Red 128 by VITABR13 in the present study compared to Lactose and Yeast extract in another similar study for decolorization of Everzol Red RBN [52].

[V] CONCLUSION

Although decolorization is a challenging process to both the textile industry and the wastewater treatment, the result of this findings and literature suggest a great potential for bacteria to be used to remove color from dye wastewaters. Interestingly, the bacterial species used in carrying out the decolorization of Azo dye Acid Red 128 in this study was isolated from the textile dye industry waste effluent. The bacterial strain *Bacillus endophyticus* VITABR13 showed decolorizing activity through a degradation mechanism rather than adsorption. This observation has established that the bacteria are adaptive in nature and can degrade contaminants. The ability of the strain to tolerate, decolorize azo dyes at high concentration gives it an advantage for treatment of textile industry wastewaters. However, potential of the strain needs to

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be demonstrated for its application in treatment of real dye bearing wastewaters using appropriate bioreactors.

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DEGRADING QUALITY OF ABSTRACTS AND PRESENTATIONS AT SCIENTIFIC CONFERENCES

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DEGRADING QUALITY OF ABSTRACTS AND PRESENTATIONS AT SCIENTIFIC CONFERENCES

The quality of abstracts and presentations at many scientific conferences calls for immediate attention and upgrade. Scientists who value quality would easily pick out how many scientific societies/associations might have being putting more interest on the quantity (numbers) rather than the quality of both the abstracts they accept and the presentations made at the scientific sections of their conferences. Similar concern about such low abstracts qualities can be inferred (indirectly) from the publications by Scherer et al. [1], Pless and Rivara [2], Gandhi and Gilbert [3], etc. These poor research, abstracts, and presentation qualities essentially prevent majority of those studies from being finally published in peer review journals [4].

Many conferences' organizing committees (COCs) do not even maintain the formats of abstracts, let alone the quality of the studies being presented in the abstracts. Some abstracts would be too long; some would be structured, others would not; worst still, some abstracts would be so irrelevant to the theme of the conference; many others would be full of errors that would normally have been corrected (or the abstracts would have been rejected) if, at least, the abstracts were truly reviewed.

The menace of these extremely poor quality would be that many students who attends such conferences with the hope of learning may eventually learn very little or even nothing; so others who are attending such type of conference for the first time might be tempted to believe that scientific conferences are always that worse. Another problem from such pitiable quality of abstracts and presentations is that participants who did just fairly well in their presentations might start to believe that they had performed excellently well, since sense of satisfaction is often relative.

Putting an end to this falling quality would depend on an understanding of the sources of the problems. In reality, some COCs give little or no review to the abstracts they receive prior to finally accepting such abstracts; some would receive abstracts even up to the last minute the abstracts are supposed to be sent to the press, giving them extremely small (or, sometimes, no) time for review. A number of COCs who claim to review the abstracts do not blind the reviewers and often give an unimaginably large room for bias. Worst still, many of these "unworthy" abstracts and studies would be presented at such conferences with minimal (or even, no) criticism: perhaps because the chairmen and the moderators of the sections are bias, and/or because the time allocated for questioning and deliberation on each presentation is often too small.

Scientific communities should fight these low and falling standards of the A to Z of conferences activities. To achieve this, it may be important that COCs pay a lot of attention to (1) thoroughly reviewing abstracts prior to accepting; (2) double-blinding the review process; (3) requesting the assistance of some international experts in the review process; (4) inviting international (or, at least, third-party) experts as moderators or chairmen of scientific sections; and (5) giving more time for deliberation and questioning at scientific sections. Or else, the quality of abstracts and presentations at many scientific conferences (as well as the goals of such conferences) would keep going down the drain.

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