ECOLOGY AND EPIDEMIOLOGY OF ATHLETIC PROTECTIVE MOUTHGUARDS

By

JAMES W. BULLARD

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Southwest Missouri State University

Springfield, Missouri

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ECOLOGY AND EPIDEMIOLOGY OF ATHLETIC PROTECTIVE MOUTHGUARDS

Thesis Approved:

Robert A Conrad

Thesis Adviser

David John

Gary K Watson

Alfred Larkozzi

Dean of the Graduate College

PREFACE

This study is a continuation of the work being done by Dr. Richard T. Glass, D.D.S., Ph.D., and Dr. Robert S. Conrad, Ph.D., of the Oklahoma State University – Center for Health Sciences faculty on the microbiology of toothbrushes, dentures, and athletic mouthpieces. I thank them both for letting me be a member of the team. It has been an enlightening experience to look at the microbes that inhabit these supposedly benign oral appliances! Also, I thank Dr. Glass and Dr. Conrad, (my advisor), for letting me, as my thesis, explore more deeply the microbial ecology of oral appliances.

Also, I sincerely want to thank all the faculty and staff of the Basic Health Sciences and Graduate college of OSU-CHS for allowing, supporting, assisting, guiding and enabling a very non-traditional graduate student (politically correct (PC) term for senior citizen) to take another major step forward toward a major life goal.

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NOMENCLATURE

AMI	Antibody Mediated Immunity
APC	Antigen Presenting Cell
API	Analytical Profile Index
BHI	Brain Heart Infusion Broth
CD-4	T-helper cell
CMI	Cell Mediated Immunity
DC	Dendritic Cells
EVA	Ethylene Vinyl Acetate
FMG	Football Players' Mouthguards
GF	Boone T. Pickens Football Field
HIV	Human Immunodeficiency Virus
HMG	Hockey Players' Mouthguards
IL	Interleukin
LR	Locker Room
MHC	Major Histocompatibility Complex
MS-I	1st Year Medical Students
MYP	Mannitol-Egg Yolk –Polymyxin Agar
OSU	Oklahoma State University

OSU-CHS	Oklahoma State University – Center for Health Sciences
PF	Practice Field
SBA	Sheep Blood Agar plate
SDA	Sabouraud Dextrose Agar
SF	Soccer Field
sIgA	Secretory Immunoglobulin A
T	Thymus derived
Th-1	Cell Mediated Immunity T-helper pathway 1
Th-2	Cell Mediated Immunity T-helper pathway 2
TNF	Tumor Necrosis Factor
TPI	Toxin Profile Index
UD	Used Dentures

CHAPTER I: INTRODUCTION

BACKGROUND: The long-term effects of wearing athletic mouthguards are unknown and may not be as benign as presently thought. Athletic mouthguards, though simple and inexpensive devices, are, nevertheless, essential pieces of equipment for the protection of the mouth and teeth as well as for some possible protection against concussions [65]. Considering all these positive effects, little attention has been paid to the possible deleterious effects that the wearing of these oral appliances may have on oral cavity microbial ecology. Since there is a close and complex relationship between oral cavity ecology and systemic health, it is possible that the alteration of oral ecology by the wearing of these appliances may have an important, and previously unappreciated, effect on the overall health of the wearer.

The normal oral cavity microbial ecology is quite complex and highly dynamic. It is composed of interrelated populations of viruses, bacteria, fungi and protozoa. Microorganisms are in constant transit into and out of the oral cavity, while populations of species are in constant flux. In this environment each microbial species seeks to find a niche for itself and its progeny. When an oral appliance (i.e. mouthguard) is placed in the oral cavity, it is quickly contaminated with indigenous microorganisms [29]. If the contamination simply reflects the population of microorganism normally found in that mouth, the wearing of a mouthguard should not create a problem. However, if the

wearing of a mouthguard gives a selective advantage to a particular species, either an existing species or a species introduced with the mouthguard, it may result in an increase of that species. This increase of a specific species may result in a significant alteration of the oral cavity microbial ecology. If the species is a frank pathogen, the resulting effect on health may be obvious. However, due to the complex relationships between oral cavity ecology and systemic health, significant changes in oral cavity microbial ecology may be extremely hard to correlate with changes in systemic health.

For reasons of health, safety and cosmetics, an ever-increasing number of people are wearing mouthguards, composed of a variety of synthetic materials. They provide a new environmental niche for microbial growth when worn in the oral cavity and can affect an alteration in the oral cavity microbial ecology. Despite these considerations, except for an occasional hypersensitivity reaction, mouthguards seemingly have a benign effect on the wearer's health. However, there is significant evidence to the contrary. In a recent article in Sports Illustrated, William Nack [64] reported on the long-term health status of retired pro-football players. He relates that many cases of later-life multiple health problems were due primarily to injuries received many years earlier during their pro-football careers. According to this 1994 survey, the most commonly reported health problem among retired football players was osteoarthritis, including some forms of inflammatory arthritis, which are known to have an autoimmune etiology. The long delay between injury and arthritis supports the hypothesis of an autoimmune etiology. Autoimmune diseases are the result of inappropriate or over-reactive immune responses, some types of which are induced by microorganisms [73]. Therefore it follows those

alterations of the oral cavity microbial ecology induced by the wearing of safety mouthguards may have played a significant role in the pathogenesis of these serious later life health problems.

Isolation and identification of all microorganisms from the complex oral cavity ecological system would be an overwhelming task. The oral cavity flora (and fauna) has been estimated by molecular biology techniques to contain over 500 different species from approximately 100 different genera [69]. It is highly unlikely that any one individual will have all the approximately 500 different species. Moore et al. [62] estimated that the average healthy mouth harbors 40-60 different species of organisms capable of being grown on artificial media using currently available techniques.

The fastidiousness and complex interactions of the "normal" oral microbial flora makes a comprehensive identification of these microorganisms an impossible task. In order to monitor the effects of wearing an oral appliance on the oral cavity microbial ecology this study used two groups of "sentinel" organisms, yeast and the bacterium, *Bacillus cereus*. These two organisms functioned as sentinel organisms because they are comparatively easy to culture and identify so that changes in the populations of these two organisms are relatively easy to detect and relate to ecological changes in the nasopharyngeal cavity. Furthermore, some species of yeast and *B. cereus* are usually considered to be soil microbes and may be used as indices of external contamination.

ATHLETIC MOUTHGUARDS: There is no doubt that athletic mouthguards have made substantial contributions to the health and well being of athletes; substantial evidence demonstrates the benefits of wearing safety mouthguards in a number of sports [65]. These devices are currently being used in many organized sports and have significantly reduced the risk of injury to the mouth and teeth. They are also worn to reduce the possibility of concussions due to blows to the jaw [6]. Boxers first used athletic safety mouthguards in the 1930's. Today, children begin wearing athletic mouthguards while participating in sports at the 3rd or 4th grade level. People also wear similar mouthguards for non-sporting events such as the prevention of snoring and teeth grinding during sleep. This increasing use of mouthguards for athletic and medical considerations has resulted in an increase in the population of people wearing these oral appliances.

Athletic mouthguards are available in four styles based on cost. They range from the cheapest, least protective, to the most expensive, most protective[65]. In the same order they are: stock, boil and bite, custom fabricated and heat/pressure laminated. All styles are available in a variety of colors with the latest trend being mouthguards that match the team colors. Plastic is the major component of most of the safety mouthguards. The polymerization process of making mouthguards produces gases that ultimately escape to the surface leaving pores on the surface and porosities in the depths of the plastic [20]. These plastic surfaces whether found superficially or in the depths, are charged surfaces. These ionized plastic surfaces are attractive adherence sites for the proteins and glycoproteins that make up the external surfaces of most microorganisms.

Most commercially available plastic mouthguards are composed of ethylene vinyl acetate (EVA)[87], which is also used in a variety of medical devices. EVA is used because it is relatively non-allergenic and is neutral towards biological systems. EVA is widely used in catheters, prosthetic implants, or other medical devices. It is, however, not without some medical risks. Its charged surface provides a convenient platform for the adhesion of microorganisms, such as *B. cereus* [4] and *Candida spp.* [18], contributing to the pathogenic process of these organisms.

ORAL CAVITY ECOSYSTEM: Oral microbial ecology encompasses the interrelationships among living organisms and their relationship to the physical environment of the oral cavity. The oral cavity ecosystem consists of all the microorganisms and host cells of the oral cavity, the surrounding "enclosing" environment and the interactions among all these components. The oral cavity consists of the buccal cavity, teeth, gums, tongue, hard and soft palates and saliva. As in all ecosystems, the oral cavity environment consists of two components, the abiotic and biotic. The abiotic, or non-living physical and chemical components of the oral cavity include energy (heat), gases such as CO_2 and O_2, liquids (saliva) and inorganic solids (dental enamel). The biotic, living components consist of all the microorganisms and the host cells. The establishment of the oral cavity ecosystem is an ongoing process of microbial colonization that involves the interaction of the microbes and their physiological process with that of the host's cells and their physiological processes [49].

The microorganisms that make up the "normal flora" of the oral cavity are components of a complex ecology that is quite likely unique to each individual. The microbial inhabitants of the oral cavity contain many diverse non-pathogenic and pathogenic species. The microbial population in each individual's oral cavity will vary as a function of differences in biotic and abiotic environmental factors. As a result each individual's oral cavity will have a different ecology at different sites and niches within their oral cavity.

The oral cavity is an open system. Microorganisms normally enter the oral cavity through the mouth or nasal openings and exit into the gut via the esophagus. The gut, starting with the stomach, is a hostile environment for most oral bacteria. One major function of saliva is to keep microorganisms moving through and out of the oral cavity. The majority of the microorganisms in the saliva are in transit to the gut, either just passing through or having been forcibly removed from a surface. Colonization of the oral cavity selects for microorganisms that are able to adhere to surfaces in such a manner that they are not readily removed by the normal flow of salivary fluids [49]. Microorganisms that survive in the oral cavity are not the same as those in the gut as shown by the studies of Martin et al. [55].

Microorganisms can exit the oral cavity by aspiration, which may have serious consequences. Nikawa et al. [66] state that aspiration of microorganisms from denture plaque into the respiratory system can expose the host to infections by microorganisms not normally found in the oral cavity or respiratory system. It follows that there is a high

probability that during a football game football players are aspirating microorganisms from the mouthguard.

BIOFILMS: A mechanism that enables many microorganisms to colonize the oral cavity is the formation of a biofilm. Oral cavity biofilms, (e.g. plaques), are very complex communities composed of many different species, with each species playing a different role in the community. The spectrum of organisms and the roles (niches) that they fill in the biofilm environment depends on a myriad of environmental factors. For example, the biofilms that form on the dental enamel are different from those that form on mucosal surfaces, and the biofilms that form in subgingival crevices will be different than those which form on subgingival surfaces [14]. Due to the nature of plastic mouthguards, the biofilm that form on the plastic oral appliances will be different from those that form on natural oral surfaces.

There are several important aspects of oral cavity biofilms. Biofilms regulate their own internal oxygen concentrations. Anaerobic conditions within biofilm depths are generated by the respiration of facultative microbes that eliminate molecular oxygen from the depths of the biofilm. The resulting anaerobic conditions allow for much higher population levels of anaerobic bacteria in the oral cavity [53]. Studies suggest that the interspecies transfer of genetic material is facilitated in oral cavity biofilms [39]. For example, Roberts et al. [74] demonstrated the transfer of a transposon coding for tetracycline resistance from *B. subtilis* to an oral cavity *Streptococcus*. In addition, Gilbert et al. [27] has shown that bacteria growing in biofilms

can be a thousand fold more resistant to antibiotics than the same bacteria growing in a pure culture.

The oral cavity ecology may be summarized as a competition between oral cavity microorganisms attempting to establish residence in a rich but somewhat hostile environment, with the host attempting to keep the oral cavity biofilms under control. It therefore follows that the oral health (and general health) of the host depends on establishing and maintaining a stable, symbiotic, balanced relationship among the microorganisms, microbial biofilms and the oral cavity environment. Anything that significantly alters any component of this complex ecosystem can upset the delicate balance and result in both oral and systemic pathogenesis [54].

ORAL CAVITY ECOLOGY AND SYSTEMIC HEALTH: The relationship between oral microbial ecology and systemic health is complex. The previously described oral microbial ecology is a delicate balance that is easily upset. It is generally accepted that changes in the ecological balance of organisms in the oral cavity can adversely affect the health of the oral cavity. And that changes in the general systemic health can alter the homeostatic balance in the oral cavity microbial ecology. However, recent studies have found that alteration in the oral cavity microbial ecology can directly affect systemic health [48].

Xerostomia or dry mouth is an example of an alteration in systemic health that results in changes in the oral cavity microbial ecology. Xerostomia may be a side effect of

numerous common drugs, radiation therapy to head and neck, and chemotherapy. Systemic diseases such as autoimmune diseases, diabetic nephritis, and various neoplasms can cause xerostomia. Xerostomia can also result from nutritional disorders, stress and HIV infection [56]. Xerostomia results from impaired saliva production, which changes the physical environmental of the oral cavity. Saliva is the aqueous mixture of secretions from the oral cavity mucus and serous salivary glands. One function of saliva is to control the growth of oral flora by altering adherence, which subsequently aids in the removal of microorganisms from the oral cavity [19].

Examples of altered oral microbial ecology affecting systemic health include cardiovascular disease [25], and peripheral vascular disease [23]. McGraw [58] has suggested a link between periodontal pathogens and pre-term delivery of low-birth-weight infants. Other workers have linked autoimmune disease with the release of lipopolysaccharides from gram-negative bacteria in the oral cavity [89].

There are a myriad of things humans do routinely that upsets the balances in the oral cavity microbial ecosystem that may lead to both oral and systemic disease. Natural processes such as eating and drinking can introduce new microorganisms, dislodge biofilms and alter saliva flow, resulting in shifts in the balances of microbial ecology. Unnatural processes such as tooth brushing, flossing, or gargling result in disturbances in oral microbial ecology. In addition, systemic activities such as fever, or the administration of antimicrobials [78] or other drugs all have the potential of disrupting the balances in the oral cavity microbial ecology. Placement of an oral appliance (i.e.

dentures, athletic mouthguard or pacifier) in the oral cavity for extended periods of time not only has the potential of importing new species of microorganisms but also introduces a new surface for the formation of biofilms, leading to the alteration of microbial populations and possible pathogenesis.

ORAL APPLIANCES, MICROORGANISMS AND ORAL ECOLOGY: The oral cavity regulates microbial populations by a variety of both innate and adaptive mechanisms. Selective innate physical factors for specific microorganisms include the mucus layer, saliva flow, pH and temperature. Selective innate chemical factors include salivary IgA (sIgA), lysozymes, lactoferrin and complement [24]. Innate cellular components such as neutrophils are active in the selection of specific microorganisms.

The construction and function of the oral cavity is of itself a physical control factor in microbial selection. Most of the surfaces of the oral cavity are lined with non-keratinized stratified squamous epithelium. The dorsal surface of the tongue, which is frequently in contact with the hard palate, and the outer surface of the gingivae (gums) are covered by a thick keratinized stratified squamous epithelium. The teeth are covered by non-cellular mineralized enamel. The non-keratinized stratified squamous epithelium that lines the wet areas of the mouth is composed of living cells. This area differs from the keratinized stratified squamous epithelium in that the uppermost layer of the keratinized epithelium is composed of dead cells. The intact interior layer of living epithelial cells forms a barrier that prevents the oral cavity microorganisms from entering the host's body [57].

In order for a microorganism to become a part of the flora of the mouthguard or mouth it must first adhere to a surface. The adherence of microorganisms to a surface in the oral cavity is not a random event. A major selective factor is the relative hydrophobicity of the oral cavity surfaces and the microorganisms. Hydrophobicity of a surface area is the result of the composition and distribution of partial charges on the surface. Relative hydrophobicity can be measured by the tendency of the surface to partition between polar and non-polar liquids. Increasing the hydrophobicity of both the surface and the microorganism results in tighter adherence between the two (likes attracting likes). This relationship is similar whether the surface involved is a natural oral cavity surface, or the surface of a mouthguard or the surface of an indwelling catheter.

Differences in hydrophobicity are also important in the interactions between microorganisms and the host's immune cells. Host phagocytic cells such as neutrophils engulf and destroy microbial cells. Other host phagocytic cells such as macrophages also engulf and destroy bacterial cells but have the capability to degrade the ingested bacterial cells and sequentially present bacterial protein to lymphatic cells for further processing as antigens. Phagocytosis is dependent on the immune cell's capability to attach to microorganisms. Microbial cells possessing a hydrophobicity that prevents them from being phagocytized have some measure of protection from the host's immune system, but these same microbial cells are subject to removal from the oral cavity by the normal flow of saliva. This further illustrates the complexity of the relationships between microorganisms and the oral cavity ecosystem.

The innate defensive mechanisms of the oral cavity are important in the control and selection of oral microorganisms, but the fine-tuning of the process must rely on the adaptive defensive mechanisms of the host's immune system. The normal healthy oral cavity allows for many different species of microorganisms to live in the oral cavity. Although these organisms grow in the oral cavity, penetration of microorganisms through the epithelial cell barrier results in a strong and rapid defense mounted by the immune system. Thus systemic health depends on the competent operation of both the innate defense mechanisms and also on a healthy, intact immune system. Also, when an alien microorganism enters the oral cavity, the host's defense systems must be able to react and control or eliminate the offensive microbial invader from the oral cavity.

The host immune response is an adaptive system based on a series of cells and cellular responses. To this end immune system cells must be able to recognize alien microorganisms, alert the system to the presence of the intruder, and choose the appropriate response. The immune system must then mount a rapid response against the offending microorganism and, at the proper time, dampen the response to normal levels.

The first oral cavity cells that come in contact with microorganisms from the outside are those of stratified squamous epithelium. Attachment to the host epithelial cell by a microbe or damage by microbial exo-proteins to the host epithelial cell can initiate a recognition cascade that results in release of pro-inflammatory cytokines, including interleukin (IL-1) and tumor necrosis factor (TNF) [36]. The pro-inflammatory cytokines recruit and activate immune system cells in the submucosa, including dendritic cells

(DC). DCs are derived from monocytes and are one type of antigen presenting cells (APC) of the immune system [80].

The DC presents an antigen to a thymus (T) derived lymphocyte. It is at this point that the "decision" is made as to which microorganisms are permissible in the oral cavity and at what population levels, and which organisms are recognized as foreign and must be eliminated. The APC is able to internalize a microorganism, or its exo-protein, process the protein material, and convert it into antigen. The antigen is then exported to the surface of the APC and aggregated with the major histocompatibility complex (MHC) on the surface of the APC. The antigen-MHC complex on the surface of the APC is then presented to the T-helper cell (CD-4). The interaction among the antigen, the MHC and the CD-4 cell provides the information that determines the immune system response [80]. Although the internal environment of the host may modulate the response, the response is probably genetically programmed at the conception of the host, and only needs the exposure of the specific antigen to set the immune response in motion.

The immune system has two pathways by which it can respond to an antigenic stimulus: the cell mediated immunity (CMI or Th1) pathway or the antibody mediated immunity (AMI or Th2) pathway. When an antigen is presented to a naive CD-4 cell, the immune system will respond with one or the other, but not both of these pathways [22]. The selection and expression of pathways is mediated through cytokines. Certain cytokines, which are chemical messengers produced by host cells, are associated with each pathway.

TNF is associated particularly with the CMI pathway, while IL-4 and IL-6 are associated with the AMI pathway [75].

One AMI response that is particularly important in the oral cavity is the production of secretory Immunoglobulin A (sIgA). The sIgA is a "learned" specific response of the immune system, in which the specificity resides in the immunoglobulin portion of the sIgA molecule. The secretory component of sIgA allows it to pass through the epithelial cell and to function in the mucous layer without being destroyed by digestive enzymes. The sIgA is able to bind microorganisms and agglutinate them and/or block them from attaching to oral cavity surfaces. The secretory components also allow the sIgA to function without activating the inflammatory complement cascade. Individuals deficient in sIgA are at increased risk of respiratory infections [30].

While the immune system protects the host from attacks by microorganisms, it allows and controls the continual co-existence of approximately 10^{13} microbial cells per person (approximately ten times the number of microbial cells as human host cells) [79]. The regulation of such a system is extremely complex. There are multiple ways the system can be compromised with infection with the Human Immunodeficiency Virus (HIV) being an example. HIV infects the pivotal CD-4 lymphocytes, and cripples the whole immune system. This crippling of the CD-4 cells allows a myriad of opportunistic infections and malignant cells to proliferate. There are also "natural" integral ways that the immune system can loose homeostasis. Overreaction or failure to shut down can result in pathogenesis. Loss of control of Th1, the CMI pathway, results in

immunopathology that can be summarized as organ-specific autoimmunity. Diabetes mellitus type I being a prominent example. Loss of control in the Th2, the AMI pathway, can result in allergy or broad-spectrum autoimmunity with lupus erythematous being a prime example [47].

YEAST AND THE ORAL CAVITY: The first "sentinel" group of organisms used in this study is yeast. The yeast group was considered because of the relationship between yeast and the host's oral and systemic health. In the immune-competent human host *Candida albicans*, the most commonly isolated yeast species, is a component of the normal flora of the human oral cavity. In the immune compromised, *C. albicans* can become a pathogen, causing diseases ranging from fairly innocuous oral thrush to life-threatening candidemia [35].

C. albicans can be found in the oral cavity of approximately 40-60% of a normal healthy human population [34]. Moalic et al. [61] in a study of 353 French students found 58.6% positive for *Candida*, of which 93.6% were identified as *C. albicans*. In a study of infants, Darwazeh et al. [13] found that 48% were colonized by *C. albicans*. He also noted that there was a higher colonization rate in infants who routinely suck on pacifiers. Pacifiers are yet another type of plastic oral appliance that may have an affect on the oral microbial ecology and subsequently on systemic health.

In a study of advanced cancer patients, Davis et al. [15] found 66% of the subjects were positive for oral cavity yeast. Only 46% of the isolates were *C. albicans*, 18% were

Torulopsis, 5% were *C. dubliniensis* and 5% were other yeast. (*C. dubliniensis* is a recently described species closely related to *C. albicans* that was first identified in Dublin, Ireland in 1995 [82].) This change of yeast species is a result of alteration in the oral cavity environment, either the biotic or abiotic. They suggest in their study this was due to an increased percentage of the population wearing dentures as well as due to decreased saliva flow due to the cancer or cancer treatment. This change in the yeast populations is evidence of the alteration of oral cavity microbial ecology by factors in and outside the oral cavity.

C. albicans is the most common etiological agent of oral thrush. Thrush is usually a reflection of the weakening of the overall immune response of the host [2]. Prior to the advent of the HIV era, oral thrush was simply the most common mycotic infection. Currently, thrush is considered a sentinel symptom for HIV-infected and other immune-compromised patients.

Although *C. albicans* infections typically reflect an underlying immune system defect in the host, there may also be changes in the *Candida* organism related to its pathogenic ability. One pathogenic factor of yeast is adhesion factor; which enables *Candida* to attach to host epithelial cells [42]. The oral appliances used in this study have plastic as a major component. The surface of these plastic devices is different from any of the natural surfaces in the oral cavity in that the plastic surface whether superficially or in the porosities is charged. These charged surfaces attract charged molecules such as proteins and glycoproteins to initiate adhesion. Yeast cells have proteins and glycoproteins on

their cell surface, which can adhere directly to the plastic surface or indirectly to other proteins or sugars attached to the plastic surface [11]. Other organisms, such as oral bacteria, can become involved in the formation of biofilms on the surfaces and depths of the plastics oral appliances. These biofilms can be quite complex and are selective factors involved in the relative ability of different yeast to adhere to oral appliances.

The pathogenicity of *C. albicans* includes the yeast's ability to produce exotoxins, including acid proteinase [59]. The relative ability of a strain of *C. albicans* to produce acid proteinase is directly related to its adhesion capability [38]. The release of exotoxins from *C. albicans* stimulates the oral cavity epithelial cells to generate and release predominately pro-inflammatory cytokines [81]. The production of these cytokines by the host's oral epithelial cells will have an effect on the overall general health of the host. The relative ability of various strains of *C. albicans* (and other genera of yeast) to induce cytokine production by host cells still remains unclear [67]. It is yet to be determined if the simple colonization by other genera of yeast induces the host to produce inflammatory cytokines.

***B. CEREUS* AND THE ORAL CAVITY:** The bacterium, *Bacillus cereus,* was chosen as the other "sentinel" organism in this study because it is ubiquitous in soil and yet is seldom found in human nasal or oral cavities. *B. cereus* is usually considered to be a saprophytic soil bacterium with low pathogenicity. It is a gram-positive spore-forming rod that is comparatively easy to identify on a gram stain. When grown on Trypticase Soy Agar with 5% sheep blood (SBA) *B. cereus* colonies are hemolytic with a

characteristic colonial morphology. The spores of *B. cereus* are relatively resistant to heat. These characteristics make *B. cereus* comparatively easy to isolate and identify.

The *Bacillus* genus is composed of a large group of gram-positive, spore-forming rod shaped bacteria. *Bacillus* species range from aerobic to facultative anaerobic with respect to molecular oxygen tolerance. *Bacillus* species can be separated from the closely related genus *Clostridium* by the fact that *Bacillus* can produce spores in the presence of molecular oxygen, while *Clostridium* cannot. Also, *B. cereus* and most other *Bacillus* species are catalase positive while the anaerobic *Clostridium* species are catalase negative [84].

The *Bacillus cereus* group is a closely related group of species within the *Bacillus* genus. The *B. cereus* group currently includes six species: *B. cereus*, *B. thuringiensis*, *B. anthracis*, *B. mycoides*, *B. pseudomycoides*, and B. *weihenstepanesis*. All six species share a great deal of genetic and phenotypic similarities. How many and which of these species are actually separate species is a matter of debate. Some or all of these species may actually be subspecies or variant strains of *B. cereus*. For example, some microbiologists consider *B. anthracis* to be a variant strain of *B. cereus* [37].

Despite these similarities there are some significant biochemical differences among the members of the *B. cereus* group. *B. cereus* and *B. thuringiensis* normally produce β-lactamase; while *B. anthracis* strains do not have this capability. *B. thuringiensis* strains have the ability to produce crystalline parasporal inclusions, which are used

commercially as insecticides. None of the other members of the *B. cereus* group are thought to be capable of producing these unique inclusions. Only a few strains of *B. anthracis* are even weakly hemolytic while most of the others in the *B. cereus* group are strongly β-hemolytic. *B. weiheinstephansis* is the only one of the group that is able to grow at 7°C.

The vegetative cell of *B. cereus* is a typical gram-positive rod, 3-5μm long with a diameter of 1.0-1.2μm. The cell membrane is bounded and contained by a peptidoglycan-based cell wall. Some strains of *B. cereus* group have an S-layer which is an additional protein layer secreted by the vegetative cell that completely covers the surface of the cell [46]. *B. cereus* rapidly forms endospores under conditions of deprivation, which enables the bacterial cell to survive in harsh environmental conditions such as desiccation, starvation, heat, radiation, chemical stress and time. The spore protects the cell from these harsh environmental conditions, but is able to rapidly germinate into the vegetative form as soon as proper environmental conditions return [18].

B. cereus spores form in the mother cell without swelling of the cell wall (an identification criteria of *B. cereus* group). The spore is a multi-layered complex. The chromosome is located in the innermost compartment, which is contained within two layers of membranes. Between these two layers of membranes is a cortex composed of a specialized peptidoglycan framework and other components. A protein coat covers the outer membrane and gives the spore its durability [17]. *B. cereus* spores as well as those of *B. anthracis* and *B. thuringinesis* have another outermost layer, the exosporium. The

exosporium is composed of various complexes of proteins, lipids and carbohydrates, and is like a thin plastic bag loosely containing the spore. The exact function of the exosporium is unknown but is most likely involved in adherence and pathogenicity [12].

B. cereus spores are likely to be found wherever dust or dirt is present. In soil, *B. cereus* is considered to be a saprophyte, receiving its nutritional requirements from dead and decaying plant and animal material. The contamination of food by soil containing *B. cereus* spores is a known cause of food poisoning. *B. cereus* is also increasingly recognized as the causative agent of non-gastrointestinal infections, especially in immunocompromised patients and patients with prosthetic implants [50]. Ocular infections with *B. cereus* following trauma to the eye or associated with contact lens use is a serious acute infection that can result in blindness if not properly treated [71]. The majority of these infections can be traced to contamination by *B. cereus* spores from dirt or dust.

Jensen [43] suggested that a normal aspect of *B. cereus*' complex life cycle is an alternation between an infectious agent and an innocuous soil inhabitant. He suggests that *B. cereus* leads a slow-paced endosymbiotic life, cycling between spores and vegetative cells in the soil as well as in symbiotic relationships within invertebrate hosts. However, when given the opportunity, *B. cereus* can rapidly accelerate its population growth by going though a fast-paced pathogenic infective cycle in either an invertebrate or vertebrate host, including man.

The isolation and speciation of *Bacillus* species from nasal swabs has become a clinically relevant procedure since October 2001 when *B. anthracis* was used as a weapon of bioterrorism [10]. Kiratisin et al. [44] in a study of 689 nasal swabs from Capitol Hill personnel and 3247 nasal swabs from Brentwood Post Office personnel isolated 22 (3.2%) and 96 (3%) respectively *Bacillus* strains tentatively identified as *B. cereus*. In a study of the bacterial flora of 20 used dentures, Glass [28] isolated 1/20 (5%) strains of *B. cereus* and in a study of 15 used hockey players' mouthguards found 1/15 (7%) strains of *B. cereus*. These low levels of *B. cereus* in nasal or oral samples are probably due to the presence of *B. cereus* spores in airborne dust.

B. cereus passes through the oral cavity on a regular basis. Food will routinely contain *B. cereus* due to the ubiquitous nature of *B. cereus* in the soil and its ability to form spores that can be carried in dust. *B. cereus* normally passes through the digestive tract without causing a significant problem. Ghosh et al. [26] isolated *B. cereus* spores from 14% (100 of 711) of fecal samples from adults. The transitory passage of *B. cereus* does not usually cause symptoms severe enough to be identified as food poisoning. However, *B. cereus* strains are associated with two different types of food poisonings. The degree of food poisoning resulting from the ingestion of *B. cereus* depends on the strain of *B. cereus* involved and the number of infective organisms ingested. Emetic (vomiting) type of food intoxication is due to the ingestion of preformed toxins and typically results in symptoms within 1-4 hours. The diarrheal type of food poisoning is caused by at least three different enterotoxins. Vegetative cells growing in the small intestine produce the enterotoxins, whose symptoms appear 6-18 hours after ingestion of viable organisms [32].

Because food poisoning due to *B. cereus* is not a reportable disease, there is no record of the incidence of the disease. However, given the ubiquitous nature of *B. cereus* and its ability to move throughout the environment as airborne spores, *B. cereus* food poisoning is most likely quite common.

The emetic toxin, cereulide, is a cyclic dodecadpsipeptide. Cereulide does not lose its toxic activity at 121°C, tolerates pH of 2-11, and is resistant to pepsin and trypsin digestion. Only selected strains of *B. cereus* produce cereulide as a metabolic by-product when grown on certain foods (particularly rice). The deleterious effect on humans is caused by the ingestion of the preformed toxin [1]. Paananen et al. [68] has shown that cereulide is also toxic to human natural killer cells. Cereulide is highly lipophilic and is rapidly absorbed from the gut into the blood stream. Levels of cereulide, well below that producing emesis may still have a significant toxic effect on natural killer cells. Exposure to cereulide may have serious long-term consequences, since natural killer cells play a pivotal role in tumor surveillance and in the host's response to intracellular infections.

One of *B. cereus* enterotoxins, enterotoxin HBL, is a three-protein complex that also functions as a hemolysin. [The HBL stands for hemolytic (H), the binding component (B), and the two lytic components (L), L_1 and L_2]. When *B. cereus* strains producing enterotoxin HBL are grown on SBA plates, the three components are produced and, depending on size and concentration, diffuse into agar at different rates. When all three proteins are present in the agar in the proper proportions the sheep red blood cells are

lysed [9]. The result is an immediate surrounding of an isolated colony of *B. cereus* by a zone of non-hemolysis, surrounded by a ring of complete hemolysis, which is in turn surrounded by non-hemolysis. This pattern of discontinuous hemolysis is typical of enterotoxin HBL. Hemolysin HBL in combination with other *B. cereus* toxins is also a virulent endophthalmitic factor[8]. Another *B. cereus* enterotoxin, enterotoxin NHE, is a three-protein complex that causes a diarrheal food poisoning but does not produce hemolysis of sheep red blood cells. Selected strains of *B. cereus* produce a third enterotoxin that has a single component and does not hemolyze sheep red blood cells [7].

B. cereus, unlike its close relative *B. anthracis*, is not considered to be pathogenic when it is recovered from the oral or nasal cavity. However when *B. cereus* is isolated from normally sterile sites in the body, such as the blood, it is considered to be pathogenic [4]. Some *B. cereus* strains, under the proper conditions, can produce a wide variety of exotoxins that are pathogenic to humans. Some of these exotoxins such as cereolysin O, which is similar to streptolysin O [33], and sphingomylinase and other phospholipase C's that hydrolyze sphingomyelin, phosphatidylcholine and phosphatidylinositol, all of which are vital components of cellular membranes of mammals, are hemolytic. These exotoxins acting either alone or in combinations can produce hemolysis of sheep red blood cells (8). It is unclear which toxin(s) or combinations thereof produce hemolysis of sheep blood. However the virulence potential of these poorly understood membrane-damaging exotoxins is obvious and quite likely contributes to pathogenesis in susceptible hosts.

As previously indicated, different strains of *B. cereus* can produce a wide variety of exotoxins, which represents substantial energy costs to the bacterial cell. Many of these toxins are inducible and are produced in response to variations in the environment such as different media. This study used these variations in exotoxin production relative to media to categorize and compare *B. cereus* strains.

CHAPTER II: MATERIALS AND METHODS

SHEEP BLOOD AGAR (SBA): SBA is Trypticase® Soy Agar with 5% sheep blood (TSA II) (Troy Biologicals Inc., Troy, NY). SBA is a tryptone-based medium supplemented with 5% sheep blood that is specifically formulated to give clear and distinct hemolytic reactions. Because of *B. cereus'* distinctive hemolytic pattern, SBA was chosen as the medium of choice for this microorganism. (Figure 1)

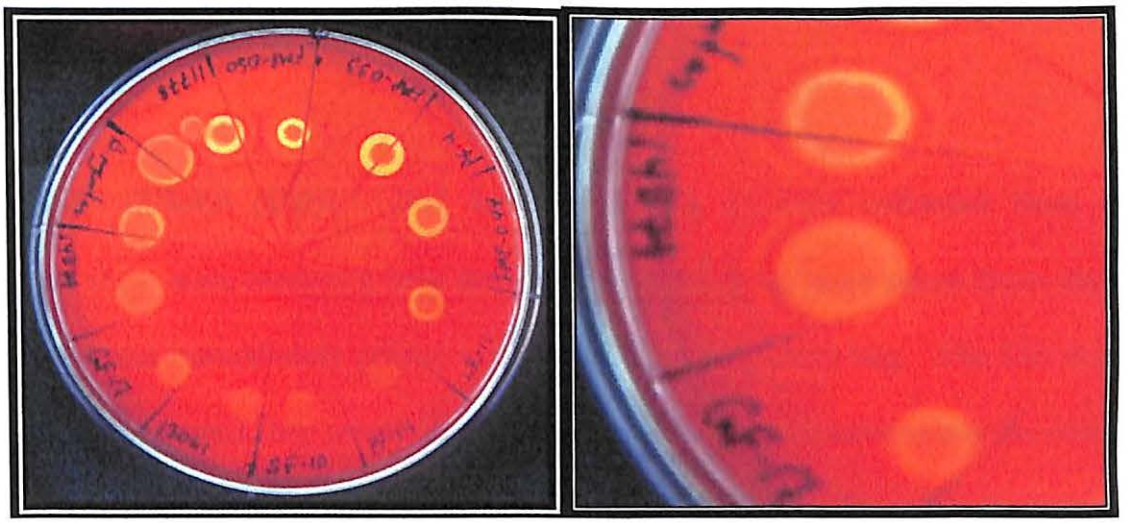

Figure 1. *B. cereus* colonies growing on SBA demonstrating various forms of hemolysis

Figure 1a. Enlarged view

SABOURAUD DEXTROSE AGAR (SDA): Sabouraud dextrose agar (Troy Biologicals Inc., Troy, NY) was the medium of choice for the isolation of yeast.

ANALYTICAL PROFILE INDEX (API) SYSTEM: Analytical Profile Index (BioMerieux Vitek, Inc., Hazelwood, MO) is a system used to identify microbial species. The API system is based on a test strip generally containing 20 miniature biochemical

tests. The results of these tests are compared for identification to the results of an extensive database of over 550 different species. Identification to the species level is thus quick and accurate. BioMerieux Vitek test systems used in this study included:

- **API 20E** Identification of *Enterobacteriacae* and non-fermenting gram-negative bacteria
- **API Staph** Identification of *Staphylococcus* and *Micrococcus*
- **API Strep** Identification of *Streptococcus* and *Enterococci*
- **API Coryne Identification** of *Corynebacterium* and coryne-like bacteria
- **API AUX 20C** Identification of yeast

USED DENTURES (UD): Used dentures (Figure 2) were collected from denture wearers as they were receiving new dentures. The dentures were taken from the mouth by a dentist and immediately placed in a sterile zip-lock plastic bag. The UD were kept in the sealed bag until they could be processed and cultured. (Institutional Review Board (IRB) Protocol-IRB# 2002003 Richard T. Glass, D.D.S., PhD. - Principle Investigator).

Figure 2. Used denture in sterile zip-lock plastic bag ready to be processed

HOCKEY PLAYER'S MOUTHGUARDS (HMG): Ice hockey players' mouthguards (HMG) (Figure 3) were collected from the Tulsa Ice Oilers, a semi-pro ice hockey team, immediately following the final ice hockey practice session. Each player placed his used mouthguard in a sterile plastic zip lock bag as he exited from the ice and entered the locker room. The mouthguard was kept in the sealed bags until it could be processed and cultured. (IRB Protocol 2002003 Amendment #1)

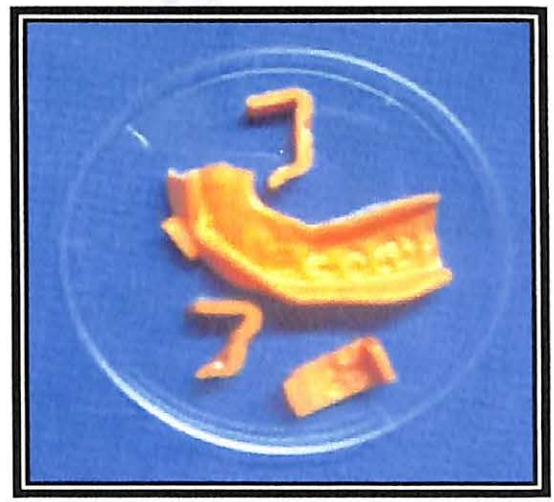

Figure 3. Mouthguard cut and ready to culture (surface and cross-section)

FOOTBALL PLAYER'S MOUTHGUARDS (FMG): The football players' mouthguards (FMG) were collected from the Oklahoma State University (OSU) football players immediately following a regularly scheduled college game. (Figure 4) Each player placed the mouthguard he had used during the game in a sterile plastic zip lock bag as he exited from the field. The mouthguards were kept in the sealed bags until the mouthguard could be processed and cultured. (IRB Protocol 2002003 Amendment #1)

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STATE ▪ OBITUARIES ▪ WEATHER

Too much exercise a bad thing?

Mouthpieces tested for germs

By DOTTIE WITTER

STILLWATER — Student and professional athletes may start paying more attention to their mouth guards in the future thanks to research being done by Dr. Melody Phillips.

Sports bras, cleats, mouthpieces have all got us that could contribute to increased infection and injury, especially as the season progresses.

"Every time you chew you have microscopic tears in your gums that act as a direct portal of entry for anything growing on the mouthpiece," Phillips said. "Cleaning or disinfecting mouthpieces may be very important for the student athletes."

Phillips, an exercise physiologist in the Health and Human Performance Program in Oklahoma State University's College of Education, along with her colleagues Drs. Robert Conrad and Tom Glass at OSU's Center for Health Sciences, have collected mouthpieces that are now being cultured to determine what might be growing on them.

"We hope to access any potential infection response that may be consequent to the wearing of those mouthpieces all season," she said.

Her major research interest is the influence of exercise on the

PHOTO PROVIDED

Dr. Melody Phillips (far left) helps gather players' mouthpieces to test for germs.

"If athletes exercise hard enough and long enough, it might actually suppress the immune system."

'the open window hypothesis,' which describes a period of time after a hard or very long exercise bout during which the body may be more susceptible to infection," she said.

"If athletes exercise hard enough and long enough, it might

and her research team is studying cytokines, the inflammatory proteins in the blood that are also associated

with cardiovascular disease, osteoporosis and diabetes.

Think of cutting your finger. The tissue around the cut becomes

Cytokines are also produced by your bone cells, and when certain conditions exist, like menopause, the bone degrading cells become more active.

"Bone degrading cells are naturally active in our body, but if they are over-stimulated, then people will experience bone loss," she said. Cytokines stimulate these bone-degrading cells.

Phillips' previous research found that just 10 weeks of resistance training for elderly women decreased the production of the inflammatory cytokines in whole blood.

The influence of exercise specifically exercise training on cytokines in elderly and at risk populations, might be of great benefit to another growing segment of the population.

"Oklahoma has a large population at risk for Type II Diabetes, the adult onset diabetes," Phillips said, citing the large American Indian population.

The cytokine most associated with insulin resistance in diabetes decreased significantly after 10 weeks of moderate to intense training, Phillips said.

"I would like to continue my line of research, to investigate the influence of resistance training in populations predisposed to having diabetes, and look at the relation

Figure 4. (Photo) Portion of a newspaper article on the collection and testing of mouthpieces (Complete article attached as appendix A)

ENVIRONMENTAL SAMPLE SITES:

- **Practice Field (PF)** is a dirt practice field located on the campus of OSU, Stillwater, Oklahoma. It is the field used by the football team as their practice field.

- **Game Field (GF)** T. Boone Pickens Field is a synthetic surface, football field located on the campus of OSU, Stillwater Oklahoma. It is the field on which the football team played just prior to the collection of the mouthguards.
- **Locker Room (LR)** The locker room in this study was the locker room routinely used by the OSU football team. It is located in the Gallagher-Iba Arena athletic building adjacent to T. Boone Pickens Field on the campus of OSU campus, Stillwater Oklahoma.
- **Soccer Field (SF)** The soccer field in this study was a lightly used dirt soccer field located on the campus of Oklahoma State University-Center for Health Sciences (OSU-CHS), Tulsa, Oklahoma.

VERO CELLS: Vero cells (African green monkey [*Cercopitecus aethiops*] kidney cells) were obtained from Dr. Earl Blewett (OSU-CHS). The vero cells were grown in Dulbecco's Modified Eagle Medium with 10% fetal bovine serum, 200 nM L-glutamine, 10,000-units/mL penicillin and 10mg/mL streptomycin [media was prepared and supplied by Tami Ross (OSU-CHS)].

MANNITOL-EGG YOLK-POLYMYXIN AGAR (MYP): Mossel et al. [63] originally formulated Mannitol-Egg Yolk-Polymyxin Agar (MYP) to isolate and enumerate *B. cereus* from foods. The MYP agar plates were prepared in the lab using the formulation and procedure as given in the Difco Manual [16]. The medium contains 1% D-mannitol (Sigma St. Louis, MO) as the source of carbohydrate, phenol red (Sigma St. Louis, MO) 0.025 g/L as a pH indicator with 1.5% agar (Acumedia Baltimore MD) as a solidifying

agent. The medium also contains 12.5ml/L of Egg Yolk enrichment 50% (Difco Detroit MI), and 25,000 units/L Polymyxin B (Sigma St. Louis, MO). Egg yolk in the media provided the lecithin and Polymyxin B inhibited the growth of most other bacteria. Bacteria that ferment mannitol produce acid, which turns the medium yellow. Bacteria that produce lecithinase hydrolyze the lecithin and form a precipitate in the media. (Figure 5)

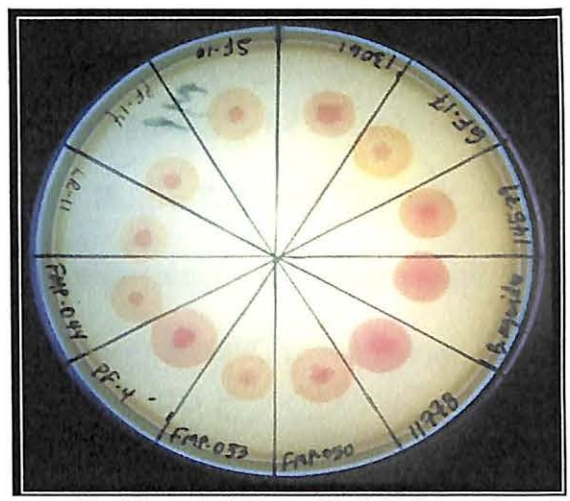

Figure 5. Mannitol-Egg Yolk-Polymyxin Agar (MYP) with *B. cereus* colonies demonstrating various amounts and types of lecithinase production

ISOLATION OF AEROBIC BACTERIA FROM MOUTHGUARDS: Cultures were made from each mouthguard by touching the exterior surface and a fresh cut cross section surface to a SBA plate. The inoculated plate was then incubated 18-24 hours at 35°C in the presence of increased carbon dioxide. (Figure 6)

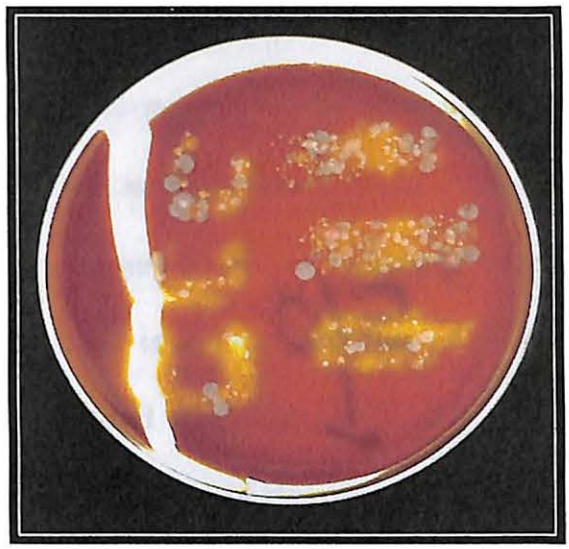

Figure 6. Contact culture (surface and cross-section) from mouthguard

After incubation a representative sample of the microorganisms was collected using a sterile swab. The swab was then rolled over one-half the surface of a fresh plate of medium (SBA), and the plate streaked for isolation with a wire loop. The inoculated plates were incubated 18-24 hours at 35°C in the presence of increased carbon dioxide. (Figure 7)

Figure 7. Mixed culture from contact plate streaked for isolation

Representatives of all colonial morphological types were transferred to a fresh medium plate, streaked for isolation and incubated 18-24 hours at 35°C in the presence of increased carbon dioxide. (Figure 8) All the microorganisms were able to grow on SBA under the conditions used for isolation. Samples of all isolated bacteria species were added to freezing medium and frozen at -70°C until they could be identified. The freezing media was composed of Brain Heart Infusion Broth (Acumedia Baltimore MD) with 10% glycerol (Sigma St. Louis, Mo).

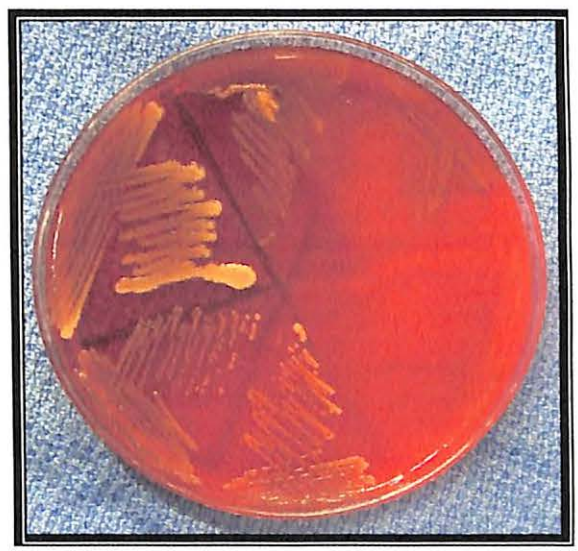

Figure 8. Isolated pure cultures of bacteria from mixed culture

IDENTIFICATION OF BACTERIA FROM MOUTHGUARDS: A specimen to be identified was removed from the -70°C freezer. Without allowing the specimen to thaw, a loop of the specimen was transferred to a SBA plate and streaked for isolation. The inoculated SBA plate was incubated for 18-24 hours at 35°C in the presence of increased carbon dioxide. Following the incubation the plate was observed and the colonial morphology recorded. A gram stain was done. The information was recorded on the

Identification of Microorganism work sheet (Figure 9) and, depending on the colonial morphology and the gram stain results, the organism was identified using the flowchart developed by Barron et al [5]. Wherever possible, the identification to the species level was done using BioMerieux Vitek API systems.

IDENTIFICATION OF MICROORGANISM

Date: _____ **Tech:** _____
Completion Date: _____

Source/History: _____

_____

_____.

GROSS COLONIAL MORPHOLOGY

Media: _____ **Thioglycollate:** _____
Colony Morphology: Size: _____ **Shape:** _____ **Elevation:** _____
Margin: _____ **Color:** _____ **Surface:** _____
Density: _____ **Consistency:** _____
Hemolysis _____
Comments:

_____

STAINS

Gram Stain: Reaction _____ **Morphology:** _____
Description: _____.

Acid Fast Stain: _____ **Modified Acid Fast** _____

Identification Procedures:

_____

_____

_____

_____

COMMENTS: _____.

Source: _____ **Organism Identification:** _____

_____

Figure 9. Worksheet used in the identification of bacteria

ISOLATION OF YEAST FROM MEDICAL STUDENTS ORAL CAVITY: First year medical students (MS-1) were sampled for the presence of yeast in their oral flora. Students' oral cavity surfaces were sampled by swabbing with a sterile cotton swab. The cotton swab was "rolled" over one half the surface of a SDA plate and streaked into four quadrants for isolation. The SDA plate was incubated for 72 hours at 37°C. Wet preparations were made of all yeast colonial types and the yeast colonies were sub-cultured for isolation to fresh SDA and grown in pure culture for identification. (IRB 2002003 Approval Amendment #2)

ISOLATION OF YEAST FROM ATHLETIC MOUTHGUARDS: FMG were collected immediately after a college football game. HMG were collected immediately following the final ice hockey practice session. Cultures were made from each mouthguard by touching the exterior surface and a fresh cut cross section surface to a SDA plate. The SDA plate was incubated for 72 hours at 37°C. Wet preparations were made of all yeast colonial types; yeast colonies were sub-cultured for isolation to fresh SDA and grown in pure culture.

ISOLATION OF YEAST FROM USED DENTURES: UD were collected from the donors. As rapidly as possible following collection, cultures were made from each used denture by touching the exterior surface and a fresh cut cross section surface to a SDA plate. The SDA plate was incubated for 72 hours at 35°C. Wet preparations were made of all yeast colonial types and the colonies were sub-cultured for isolation to fresh SDA and grown in pure culture.

ISOLATION OF YEAST FROM ENVIRONMENTAL SAMPLES: For soil samples, at each sample site approximately one gram of soil was taken from the top one-inch layer of soil and transferred to a tube containing five milliliters of sterile Sabouraud Dextrose Broth (SDB) (Acumedia, Baltimore, MD). From the GF at each sample site approximately one gram of composite material (Astroturf) was taken from the top one inch of composite material and added to five milliliters of sterile SDB. The Locker Room (LR) was sampled by wiping a dry swab over approximately one hundred square inches of surface test site. The cotton tip of the dry swab was then placed in five milliliters of sterile SDB, which was then incubated for 72 hours at 35°C. After the incubation, sub-cultures were made using a sterile loop and streaking for isolation on fresh SDA. The SDA plate was incubated for 72 hours at 35°C. Wet preparations were made of all yeast colonial types; yeast colonies were sub-cultured for isolation to fresh SDA and grown in pure culture for identification.

IDENTIFICATION OF YEAST: Pure cultures of yeast from the different sources were identified using the API 20 C AUX system according to manufacture's directions. The test is based on the yeast's ability to use 20 different carbon sources as the sole source carbon. (Figure 10) Identification of yeast was obtained by the use of the API Profile Recognition System.

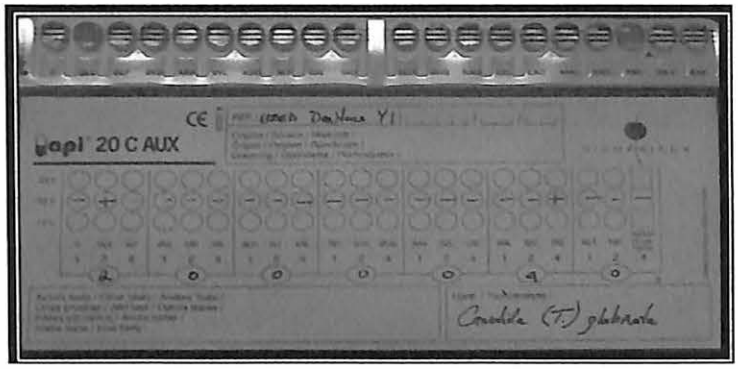

Figure 10. (Photo) API Test Strip for Yeast and record form. This is an identification of *Candida (Torulopsis) glabrata* from a used denture

ISOLATION OF *B. CEREUS* FROM ENVIRONMENTAL SAMPLES: For soil samples, at each test site approximately one gram of soil was taken from the top one inch of soil and transferred to a tube containing three milliliters of sterile brain heart infusion broth (BHI) (Acumedia, Baltimore, MD). Approximately one gram of composite material (Astroturf) was taken from the top one inch of composite material at each GF test site and added to three milliliters of BHI. LR was sampled by wiping a dry swab over approximately one hundred square inches of surface test site. The cotton tip of the dry swab was then placed in three milliliters of BHI. All the BHI samples were pasteurized by heating at 62.5°C for 30 minutes. After pasteurization, cultures were incubated at 35°C for 72 hours. Sub-cultures of each sample were made to SBA plate and MYP agar plate.

ISOLATION OF *B. CEREUS* FROM MOUTHGUARDS: FMG were collected immediately after a college football game. HMG were collected immediately following the final practice session. An approximately one-gram sample was cut from the each mouthguard and placed in three milliliters BHI. All the mouthguards BHI samples were

pasteurized by heating at 62.5°C for 30 minutes. After pasteurization, the BHI cultures were incubated at 35°C for 72 hours. Sub-cultures of each BHI sample was made by streaking for isolation on a SBA plate and a MYP agar plate.

ISOLATION OF *B. CEREUS* FROM USED DENTURES: UD were collected from each donor. A portion of the denture was held at -70°C until they could be processed for *B. cereus* spores. To test for the presence of *B. cereus* spores, an approximate one-gram sample was cut from the UD and placed in three milliliters BHI. The UD BHI samples were pasteurized by heating at 62.5°C for 30 minutes. After pasteurization, the BHI cultures were incubated at 35°C for 72 hours. Sub-cultures of each BHI sample was made by streaking for isolation on a SBA plate and a MYP agar plate.

IDENTIFICATION OF *B. CEREUS*: Gram stains were made of all cultures that were β-hemolytic on SBA, mannitol negative and lecithinase positive on MYP. Bacteria were considered *B. cereus* group if they were gram-positive bacilli and spore formers, whose spores did not extend past the sidewalls of the bacterium. All organisms identified as members of the *B. cereus* group were resistant to penicillin [84].

TOXIN PRODUCTION ON DEFINED MEDIA: Each isolated strain of *B. cereus* was inoculated into 3 ml Roswell Park Memorial Institute (RPMI-1640) (Sigma St Louis, MO) liquid media supplemented with 0.3 g/L glutamine (Sigma St. Louis, MO) and 2.0 g/L sodium bicarbonate (Sigma St. Louis, MO) and incubated overnight at 30°C with shaking. After incubation, samples were centrifuged 10 minutes at 1800 RPM.

(Beckman T-J 6 centrifuge). The supernate was then sterile filtered through 0.22 μm syringe filters (Acrodisc Pall Corp., Ann Arbor, MI). The sterile, bacteria-free, supernatant was then evaluated for the presence of five toxins.

HEMOLYSIN PRODUCTION: *B. cereus* produces several different exotoxins capable of hemolyzing sheep red blood cells in agar media. A modification of the method of Beecher and Wong [7] was used to distinguish three hemolytic conditions. This procedure uses commercially prepared SBA. To evaluate the supernatant for the presence of hemolysin, three-millimeter diameter wells were aseptically "punched" into a SBA plate and 25 μL of the supernatant to be tested was placed in a well. The supernatant was allowed to diffuse out of the well into the medium for 24 hours. The plate was then evaluated for the presence of any of the following hemolytic patterns: discontinuous pattern, clear hemolysis or no hemolysis. Discontinuous pattern is a ring of clear hemolysis some distance from the well enclosing an area closer to the well that was not hemolysed [9]. Clear hemolysis is a circular area of complete hemolysis surrounding the central well [7]. No hemolysis is demonstrated by the lack of any change in appearance of red blood cells surrounding the well. (Figure 11) The presence, type and diameter of areas of hemolysis were recorded.

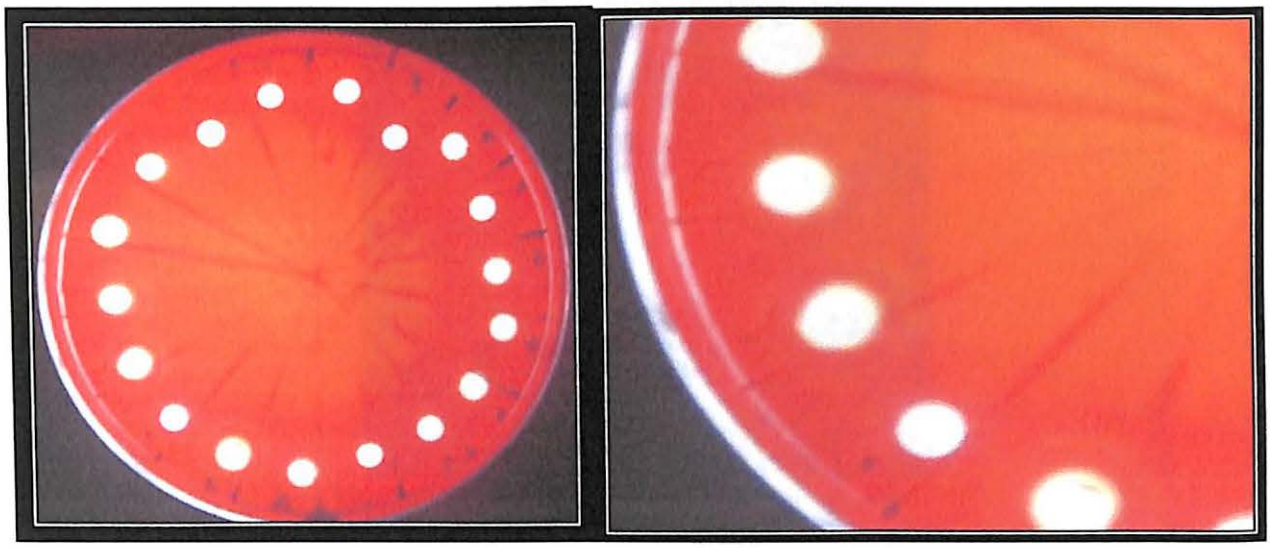

Figure 11. (left) Sheep Blood Agar with wells punched into the media and filled with sterile culture fluid from *B. cereus* cultures. Note discontinuous hemolysis in wells at approximately 7, 8 & 9 o-clock positions (Right) Enlarged view

LECITHINASE PRODUCTION: The culture supernatants were evaluated for the presence of lecithinase using the MYP that was developed by Mossel et al. [63] for the detection of lecithinase production by *B. cereus*. The procedure was modified in that three-millimeter diameter wells were "punched" into the agar media. For the test, 20 µL of the sterile test culture supernate was placed in a well. After 24 hours the plate was examined for the presence or absence of a circular area of cloudy precipitation surrounding the well. (Figure 12) The presence and diameter of the area of precipitation (lecithinase activity) was recorded.

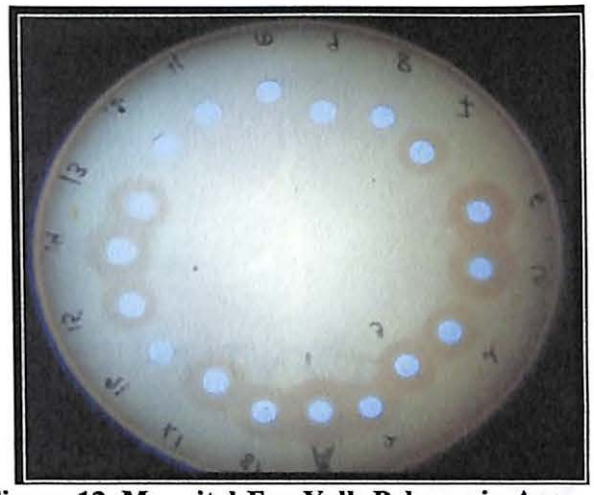

Figure 12. Mannitol-Egg Yolk-Polymyxin Agar with wells punched into the media and filled with sterile culture fluid from *B. cereus* cultures

ENTEROTOXIN PRODUCTION: The presence of enterotoxin in each supernate was evaluated using the *B. cereus* Enterotoxin-Reversed Passive Latex Agglutination test kit (BCET-RPLA, Oxoid Hampshire, England). The test was performed according to the manufacturer's directions. This is a latex agglutination test in which a visually detected agglutination indicates the production of the L_2 component of enterotoxin. (Figure 13) Some strains of *B. cereus* produce the L_2 component but do not produce the effect of discontinuous hemolysis when grown on SBA [40]. Also some *Bacillus* species, other than the *B. cereus* group have the capabilities when grown under the proper conditions to produce the L_2 factor and yield a positive result with this kit even though they may or may not produce discontinuous hemolysis [70].

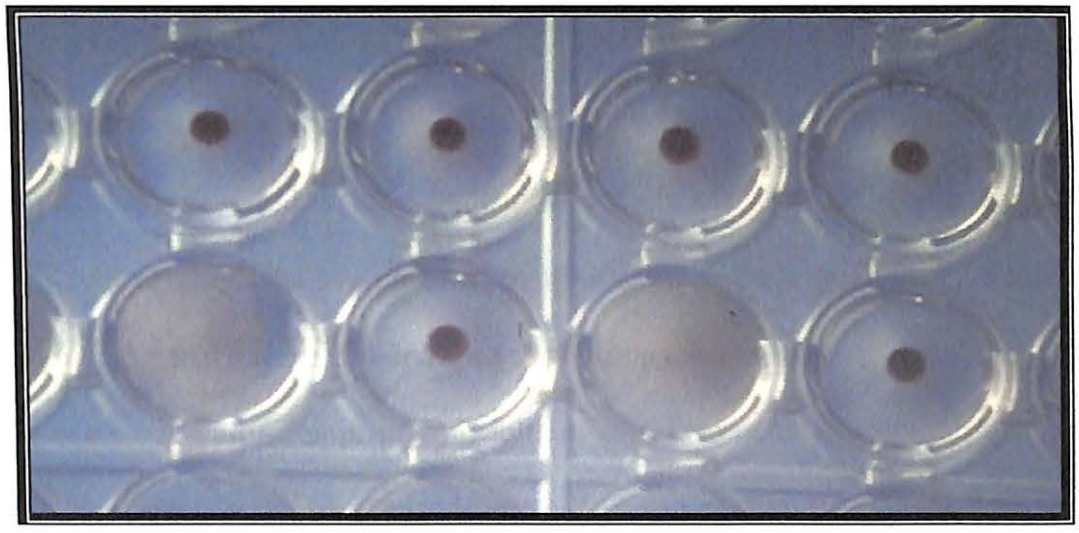

Figure 13. Enterotoxin-Reversed Passive Latex Agglutination test for the L-2 component of enterotoxin. Top row are control wells and the "button" is a negative reaction. Bottom row are the test wells and a positive reaction is the lack of the solid "button".

VEROTOXIN PRODUCTION: Using a modification of the method of Pradel [72], the presence of verotoxin was detected using a cytotoxicity assay with vero cells as target cells. To measure the cytotoxic effect, vero cells were grown in 96 well microtiter plates to approximately 70-90% confluence in 100-µL media/well ($\approx 10^5$ cells/well). One hundred microliters of the sterile culture filtrate to be evaluated was added to a test well. The microtiter plate was then incubated for two hours at 37°C in a 5% carbon dioxide atmosphere. After the two hours the vero cells were observed microscopically for cytopathic effects. The results were recorded as positive (cytopathic effect) or negative (no cytopathic effect). There appeared to be two different cytopathic effects, in one type, the cells assumed an "owl eye" appearance, and in the other type, the cells were rounded up, swollen and clear. The explanations of why two different patterns are not known, but suggests the presence of at least two different verotoxins.

TOXIN PROFILE INDEX (TPI): TPI was constructed in order to facilitate the comparison of toxin production by various strains of *B. cereus*. The TPI was modeled on the Analytical Profile Index used by BioMerieux Vitek, Inc. The production of five different toxin effects was evaluated as to whether they were positive or negative. The toxin production tests were divided into two groups, one group of three and the second with two tests. A value for positive results of one, two or four was assigned to each of the toxins, a negative results was a zero. By adding the numbers corresponding to positive reactions within each group, a two-digit number is obtained which forms the TPI. Group one was composed of discontinuous hemolysis, clear hemolysis and lecithinase. In group one, if discontinuous hemolysis was positive it was given a value of "1", positive for clear hemolysis a value of "2", positive lecithinase a value of "4", negative for any of the three was "0". The values of the first three toxins were added to give the first digit in the profile, range from 0-7. Enterotoxin and verotoxin formed group two, enterotoxin positive is "1", and verotoxin is "2", negative results are "0"s. Values for group two were added together to give the second digit with range of 0-3. The toxin profile is a combination of the two digits and range from 0|0 to 7|3 (the values have no meaning by themselves). All the *B. cereus* strains were evaluated using this system and assigned a TPI number. (Figure 14)

Figure14. Toxin Profile Index (TPI) of Bacillus cereus strains

Toxins positive for:	TPI Toxin Profile Index
None	0⊥0
Lecithinase only	4⊥0
Enterotoxin only	0⊥1
Clear hemolysis & Lecithinase	6⊥0
Clear hemolysis & Enterotoxin	2⊥1
Lecithinase & Enterotoxin	4⊥1
Clear hemolysis, Lecithinase & Enterotoxin	6⊥1
Enterotoxin & Verotoxin	0⊥3
Clear hemolysis, Lecithinase & Verotoxin	2⊥3
Discontinuous hemolysis, Clear hemolysis, Enterotoxin & Verotoxin	3⊥3
Clear hemolysis, Lecithinase, Enterotoxin & Verotoxin	6⊥3
Discontinuous hemolysis, Clear hemolysis, Lecithinase, Enterotoxin & Verotoxin	7⊥3

STATISTICAL ANALYSIS: Binomial analysis was used to estimate the probability that two populations were equivalent. For the genera of gram negative bacillus and the species of gram positive cocci the incidence in one of the populations was used as the probability in that population and the number of mouthguards positive for the organism in the other population as the number of successes. In *B. cereus* strains, the incidence of a specific TPI in the environmental samples was compared to the incidence in the FMG of that same TPI. The presence in the *B. cereus* of the test TPI was considered to be a positive (success) result.

CHAPTER III: RESULTS

BACTERIAL FLORA OF MOUTHGUARDS: Aerobic, non-fastidious bacteria were isolated from the football and hockey players' safety mouthguards. The isolated bacteria were catalogued and listed according to gram stain reaction and cellular morphology. (Table 1 and 2)

Table 1. Bacterial Species (Frequency) Isolated from FMG (n=36)
(Number in parenthesis is the number of mouthguards yielding the isolate.)

Gram Positive Cocci	Gram Negative Cocci
1. *Aerococcus spp. (2)* 2. *Aerococcus viridians (7)* 3. *Alloiococcus spp. (1)* 4. *Enterococcus spp. (5)* 5. *Leuconstoc spp. (5)* 6. *Micrococcus spp. (8)* 7. *Kocuria kristinae (5)* 8. *Pedicocccus spp. (1)* 9. *Rothia mucilaginosa (1)* 10. *Staphylococcus aureus (11)* 11. *Staphylococcus auricularis (1)* 12. *Staphylococcus capitis (10)* 13. *Staphylococcus caprea (3)* 14. *Staphylococcus chromogenes (1)* 15. *Staphylococcus epidermidis (21)* 16. *Staphylococcus haemolyticus (3)* 17. *Staphylococcus hominis (20)* 18. *Staphylococcus lentus (1)* 19. *Staphylococcus lugdunensis (3)* 20. *Staphylococcus saprophyticus (6)* 21. *Staphylococcus sciuri (2)* 22. *Staphylococcus spp. (6)* 23. *Staphylococcus warneri (4)* 24. *Staphylococcus xylosus (6)* 25. *Stomatococcus spp. (1)* 26. *Streptococcus acidominimus (4)* 27. *Streptococcus salivarius (1)* 28. *Streptococcus spp. (2)*	1. *Neisseria elongata (1)* 2. *Neisseria mucosa (4)* 3. *Neisseria sicca (1)* 4. *Neisseria spp. (3)*
Gram Positive Rods	**Gram Negative Rods**
1. *Actinomadura spp. (1)* 2. *Arcanobacterium haemolyticum (8)* 3. *Arcanobacterium spp. (4)* 4. *Bacillus cereus (16)* 5. *Bacillus pumilus (1)* 6. *Bacillus spp. (25)* 7. *Bacillus thuringiensis (1)* 8. *Brevibacterium spp. (1)* 9. *Cellulomonas spp. (2)* 10. *Corynebacterium aquaticum (2)* 11. *Corynebacterium group G (1)* 12. *Corynebacterium spp. (9)* 13. *Lactobacillus spp. (1)* 14. *Listeria spp. (3)* 15. *Rhodococcus spp. (1)*	1. *Acinetobacter lwoffi (2)* 2. *Acinetobacter spp. (7)* 3. *Actinobacillus spp. (3)* 4. *Aeromonas spp. (1)* 5. *Capnocytophaga gingivalis (1)* 6. *Capnocytophaga spp. (1)* 7. *Chryseomonas luteola (1)* 8. *Chryseobacterium indologenes (1)* 9. *Chryseobacterium meningocepticum (3)* 10. *Group NO-1 (1)* 11. *Flavimonas oryzihabitans (3)* 12. *Moraxella spp. (5)* 13. *Ochrobactrum anthropi (1)* 14. *Pasteurella pneumotrophica (7)* 15. *Pasteurella spp. (2)* 16. *Stenotrophomonas maltophilia (3)*

Table 2. Bacterial Species (Frequency) Isolated from HMG

(Number in parenthesis is the number of mouthguards yielding the isolate)

Gram Positive Cocci	Gram Negative Cocci
1. *Kocuria kristinae (3)* 2. *Staphylococcus aureus (7)* 3. *Staphylococcus cohnii (1)* 4. *Staphylococcus epidermidis (5)* 5. *Staphylococcus haemolyticus (1)* 6. *Staphylococcus hominis (1)* 7. *Staphylococcus sapropyticus (3)* 8. *Staphylococcus spp. (3)* 9. *Staphylococcus warneri (2)* 10. *Streptococcus acidominimus (1)* 11. *Streptococcus pneumoniae (1)* 12. *Streptococcus salivarius (1)* 13. *Streptococcus sanguis (1)*	1. *Neisseria spp. (8)* 2. *Bramhamella catarrhalis (1)*
Gram Positive Rods	**Gram Negative Rods**
1. *Actinomyces spp. (2)* 2. *Arcanobacter haemolyticum (2)* 3. *Bacillus cereus (1)* 4. *Bacillus spp. (4)* 5. *Corynebacterium aquaticum (1)* 6. *Corynebacterium pseudodiptheriticum (1)* 7. *Corynebacterium spp. (1)* 8. *Corynebacterium striatum (1)* 9. *Rhodococcus spp. (1)* 10. *Streptomyces spp. (1)*	1. *Acinetobacter lwoffi (4)* 2. *Acinetobacter spp. (2)* 3. *Flavimonas oryzihabitans (2)* 4. *Moraxella spp. (1)* 5. *Pantoea spp. (1)* 6. *Pseudomonas aeruginosa (1)* 7. *Pseudomonas spp. (2)* 8. *Pseudomonas stutzeri (1)* 9. *Pseudomonas fluorescens (2)* 10. *Ochrobactrum anthropi (1)* 11. *Serratia liquifans (1)* 12. *Serratia marcescens (2)* 13. *Xanthomonas maltophilia (1)* 14. *Xanthomonas spp. (1)*

Table three summarizes the results of table 1 and 2. (Table 3)

Table 3. Comparison of FMG and HMG Bacterial Isolates

Total and (average) number of isolates per mouthguard

FMG (n=36)

Total isolates	Gram Pos. Rod	Gram Pos. Cocci	Gram Negative rod	Gram Negative Cocci
259	73	137	40	9
(7.2)	(2.0)	(3.8)	(1.1)	(0.3)

HMG (n=15)

Total isolates	Gram Pos. Rod	Gram Pos. Cocci	Gram Negative rod	Gram Negative Cocci
75	15	30	21	9
(5.0)	(1.0)	(2.0)	(1.4)	(0.6)

YEAST FLORA OF ORAL CAVITY AND APPLIANCES: Yeast cultures were made from oral cavity swabs from first year medical students and contact cultures from each of the different oral appliances. (Table 4)

Table 4. Survey of Yeast Isolated from Oral Appliances
Percent positive for each of the genera of yeast

	Yeast Genera*			
Source	**Candida**	**Torulopsis**	**Rhodotorula**	**Trichosporon**
MS I	17	0	0	0
UD	50	30	0	10
HMG	40	0	0	7
FMG	3	0	17	3

*Does not include 1 isolate of *Saccharomyces* from UD and *Cryptococcus* from FMG.

Six different genera of yeast were identified. *Trichosporon* was the only genus found in all three types of oral appliances. UD had the highest number of genera, (4 genera), and was the only source of *Torulopsis* (6 isolates) and *Saccharomyces* (1 isolate). FMG were the only source of *Rhodotorula* (6 isolates); there was also 1 isolate each of *C. lusitaniae* and *Cryptococcus spp.* HMG yielded only *C. albicans* (6 isolates) (1 isolate was identified as *C. dublinsis* a very closely related species). The football mouthguards differed from the other samples in the presence of the soil yeast, *Rhodotorula*, and the low incidence of the normal oral flora yeast, *Candida*.

ISOLATION OF *B. CEREUS* FROM ENVIRONMENTAL AND CLINICAL SAMPLES: Samples were taken from environmental sites and clinical specimens and specifically cultured to reveal the presence of *B. cereus* spores in the sample. (Table 5)

Table 5. Incidence of Soil-Related Microorganisms in Environmental and Clinical Samples

Sample Source (n)	*B. cereus* Incidence(% positive)	*Rhodotorula** Incidence (% positive)
Environmental Samples		
Soccer Field (20)	20 (100%)	ND[a]
Practice Field (20)	20 (100%)	2 (10%)
Game Field (20)	15 (75%)	3 (15%)
Locker room (20)	18 (90%)	0
Clinical Samples		
Football players Mouthguard (36)	9 (25%)	6 (17%)
Used dentures (20)	1 (5%)	0
Medical Students oral swab (30)	0	0
Hockey Players Mouthguards (15)	1 (7%)	0

- All samples were concomitantly cultured for fungi such as Rhodotorula spp.
- [a] ND is not done

In the environmental samples, there is some correlation between amount of soil and the frequency of *B. cereus* isolation. All the samples from dirt fields were positive for *B. cereus* spores. The game field, which had synthetic turf as a major component, had a lower (75%) incidence of *B. cereus*; and the locker room where the dirt was only in the form of dust had an incidence of 90% positive. In the football mouthguards clinical samples, the significantly higher ($p<0.001$) incidence of *B. cereus* spores and the soil yeast, *Rhodotorula*, was noted.

SELECTION OF *B. CEREUS* STRAINS BY MOUTHGUARDS: Each isolate of *B. cereus* was grown on RPMI 1640 and the bacteria removed by sterile filtration. The sterile culture fluid was then analyzed for the presence of five exo-protein toxins. (Table 6-10) Discontinuous hemolysis, clear hemolysis, lecithinase, and verotoxin were all tests for the expression of an affect. The enterotoxin test checked for the presence of one of three proteins of the enterotoxin complex.

Table 6. TPI of *B. cereus* Strains Isolated from Soccer Field

	Toxins					
Source	DH [a] "1"	CH [b] "2"	Lecithinase "4"	Enterotoxin "1"	Verotoxin "2"	TPI [c]
SF-1	0	+	+	+	0	6⊥1
SF-2	+	+	+	+	+	7⊥3
SF-3	0	+	+	+	0	6⊥1
SF-4	+	+	+	+	+	7⊥3
SF-5	0	0	+	+	0	4⊥1
SF-6	0	+	+	+	0	6⊥1
SF-7	+	+	+	+	+	7⊥3
SF-8	+	+	+	+	+	7⊥3
SF-9	0	0	+	+	0	4⊥1
SF-10	0	0	0	+	0	0⊥1
SF-11	+	+	+	+	+	7⊥3
SF-12	0	0	0	+	0	0⊥1
SF-13	0	0	+	+	0	4⊥1
SF-14	0	0	0	+	+	0⊥3
SF-15	0	0	0	+	+	0⊥3
SF-16	0	0	+	+	0	4⊥1
SF-17	0	0	+	+	0	4⊥1
SF-18	0	0	0	0	0	0⊥0
SF-19	0	0	0	0	0	0⊥0
SF-20	0	0	0	+	+	0⊥3

[a] Discontinuous Hemolysis
[b] Clear hemolysis immediately surrounding the well
[c] Toxin Profile Index

Table 7. TPI of *B. cereus* Strains Isolated from Practice Field

Source	Toxins					
	DH [a] "1"	CH [b] "2"	Lecithinase "4"	Enterotoxin "1"	Verotoxin "2"	TPI [c]
PF-1	+	+	+	+	+	7±3
PF-2	0	0	+	+	0	4±1
PF-3	0	0	0	+	0	0±1
PF-4	0	+	+	+	0	6±1
PF-5	0	+	0	+	0	2±1
PF-6	+	+	0	+	+	3±3
PF-7	0	+	0	+	+	2±3
PF-8	0	0	0	0	0	0±0
PF-9	0	0	0	+	0	0±1
PF-10	0	0	0	+	0	0±1
PF-11	0	0	0	+	0	0±1
PF-12	0	0	0	+	+	0±3
PF-13	0	+	0	+	+	2±3
PF-14	0	0	+	+	0	4±1
PF-15	0	+	+	+	0	6±1
PF-16	0	0	+	0	0	4±0
PF-17	0	0	0	+	0	0±1
PF-18	0	0	0	+	0	0±1
PF-19	0	+	+	+	0	6±1
PF-20	0	0	0	+	+	0±3

[a] Discontinuous Hemolysis
[b] Clear hemolysis immediately surrounding the well
[c] Toxin Profile Index

Table 8. TPI of *B. cereus* Strains Isolated from Locker Room

	Toxins					
Source	DH [a] "1"	CH [b] "2"	Lecithinase "4"	Enterotoxin "1"	Verotoxin "2"	TPI [c]
LR-1	***	***	***	***	***	***
LR-2	0	0	0	+	+	0⊥3
LR-3	0	0	0	+	+	0⊥3
LR-4	0	+	0	+	0	2⊥1
LR-5	0	0	0	+	+	0⊥3
LR-6	0	0	0	+	+	0⊥3
LR-7	0	0	0	+	+	0⊥3
LR-8	0	0	0	+	+	0⊥3
LR-9	0	0	0	+	0	0⊥1
LR-10	0	0	0	+	0	0⊥1
LR-11	0	+	+	+	0	6⊥1
LR-12	0	+	+	+	0	6⊥1
LR-13	0	0	0	+	0	0⊥1
LR-14	0	0	+	+	0	4⊥1
LR-15	0	+	+	+	+	6⊥3
LR-16	0	+	+	0	0	6⊥0
LR-17	+	+	+	+	+	7⊥3
LR-18	0	0	0	+	0	0⊥1
LR-19	0	0	0	0	0	0⊥0
LR-20	***	***	***	***	***	***

[a] Discontinuous Hemolysis
[b] Clear hemolysis immediately surrounding the well
[c] Toxin Profile Index

Table 9. TPI of *B. cereus* Isolated from Game Field

Source	Toxins					
	DH [a] "1"	CH [b] "2"	Lecithinase "4"	Enterotoxin "1"	Verotoxin "2"	TPI [c]
GF-1	+	+	0	+	+	3⊥3
GF-2	0	0	0	+	0	0⊥1
GF-3	***	***	***	***	***	***
GF-4	0	0	0	+	0	0⊥1
GF-5	***	***	***	***	***	***
GF-6	+	+	0	+	+	3⊥3
GF-7	0	+	+	0	0	6⊥0
GF-8	+	+	+	+	+	7⊥3
GF-9	+	+	+	+	+	7⊥3
GF-10	0	0	0	+	0	0⊥1
GF-11	0	0	0	+	0	0⊥1
GF-12	0	0	0	0	0	0⊥0
GF-13	0	0	0	+	0	0⊥1
GF-14	0	0	0	+	0	0⊥1
GF-15	***	***	***	***	***	***
GF-16	***	***	***	***	***	***
GF-17	0	+	+	0	0	6⊥0
GF-18	0	+	+	+	0	6⊥1
GF-19	***	***	***	***	***	***
GF-20	0	0	0	+	+	0⊥3

[a] Discontinuous Hemolysis
[b] Clear hemolysis immediately surrounding the well
[c] Toxin Profile Index

Table 10. TPI of *B. cereus* Strains Isolated from Clinical Specimens

Source	Toxins					
	DH [a] "1"	CH [b] "2"	Lecithinase "4"	Enterotoxin "1"	Verotoxin "2"	TPI [c]
FMG-033	0	0	+	+	0	4⊥1
FMG-050	0	+	0	+	0	2⊥1
FMG-032	0	0	+	+	0	4⊥1
FMG-044	+	+	+	+	+	7⊥3
FMG-014	+	+	+	+	+	7⊥3
FMG-096	0	+	+	+	0	6⊥1
FMG-088	0	+	+	+	0	6⊥1
FMG-098	0	+	+	+	0	6⊥1
FMG-057	0	+	0	+	0	2⊥1
UD-001	0	+	+	+	0	6⊥1
GMP-09	0	0	+	+	0	4⊥1
CLC-2	+	+	+	+	+	7⊥3

[a] Discontinuous Hemolysis
[b] Clear hemolysis immediately surrounding the well
[c] Toxin Profile Index

Table eleven summarizes the toxin production by *B. cereus* isolated from the various sources. (Table 11)

Table 11. Percentage of Strains of *B. cereus* Isolates Positive for Selected Toxins (grown on RPMI-1640)

Sample Source (n)	Discont. hemolysis	Clear hemolysis	Lecithinase	Enterotoxin	Verotoxin
PF (20)	10	40	35	90	30
GF (15)	27	47	33	80	30
LR (18)	6	33	33	89	44
SF (20)	25	40	65	90	40
FMG (9)	22	78	78	100	22

The number of strains isolated from the environmental sources that were positive for the toxin effects of verotoxin, enterotoxin and clear hemolysin were quite consistent. The number of positives for discontinuous hemolysin was low from the locker room 5%, twice as high from the PF at 10%, and higher still from the GF at 27% positive. When the results from the mouthguards are compared to the results from environmental sources a number of differences are noted.

In order to facilitate comparisons of the isolates, the *B. cereus* strains were organized using a two-digit profile based on toxin production, the TPI. The number of strains with each profile from each of the environmental sources and FMG were recorded and than compared (Table 12).

Table 12: TPI of *B. cereus* Isolates
(Number of isolates with each of the given profiles)

TPI Profile code	PF n=20	GF n=15	L R n=18	Environ Totals n=53	FMG n=9
0⊥0	1	1	1	3	-
4⊥0	1	-	-	1	-
0⊥1	6	6	4	16	-
6⊥0	-	2	2	4	-
2⊥1	1	-	-	1	2
4⊥1	2	-	1	3	2
6⊥1	3	1	2	6	3
0⊥3	2	1	6	9	-
2⊥3	2	-	-	2	-
3⊥3	1	2	-	3	-
6⊥3	-	-	1	1	-
7⊥3	1	2	1	4	2

Out of the 32 theoretically possible profiles, only 12 were represented. All 12 of the profiles were represented, in various amounts, in the environmental samples; however, only four profiles were found in the mouthguard isolates.

Using binomial distribution analysis, the results were analyzed for the probability that the profiles of *B. cereus* strains isolated from the FMG were randomly "picked up" from the soil in the environment. The probability that any one profile would appear the number of times it does in the mouthguards just by random chance from the environment range from a low for profile "2 ⊥1" of $P<0.02$ to a high of $P<0.12$ for profile "7⊥3". All four profiles found in the mouthguards were combined and analyzed as to their randomly appearing in all nine FMG. The chance of this being a random event was $P<0.001$. (Table 13)

Table 13: Probability That Isolates are Random from the Environment
(Toxin profiles of *B. cereus* specimens)

Profile Code	Enviro. Totals n=53	FMG n=9	Probability that mouthguard strains are random from environment
2⊥1	1	2	P<0.02
4⊥1	3	2	P<0.08
6⊥1	6	3	P<0.06
7⊥3	4	2	P<0.12
Combined 2⊥1, 4⊥1, 6⊥1, 7⊥3	14	9	P<0.001

CHAPTER IV: DISCUSSION

ALTERATION OF NORMAL BACTERIAL FLORA BY ORAL APPLIANCES: The normal flora of the mouth is very rich and diverse. When an oral appliance such as a denture, athletic mouthguard, pacifier or toothbrush is placed into the oral cavity even for a short period of time it is quickly contaminated with oral microorganisms. A new athletic mouthguard, which is not necessarily sterile before it is placed in the mouth, becomes heavily contaminated with oral microorganisms after a very brief period of wear [29]. By touching a used mouthguard to a SBA a wide variety of organisms can be isolated. (Table 1 & 2) Presumably, the majority of these isolates represent the "normal" flora of the mouth. However, some interesting points arise when examining the incidence, "natural habitat" and possible pathogenesis of some of the selected genera of these bacteria. (Table 14)

Table 14. Analysis of Selected Gram Negative Bacillus Incidence (%) of mouthguards positive for specific genera of gram-negative bacillus

Genera	HMG N=15	FMG N=36	Natural Habitat/ Associated Diseases
Acinetobacter	6 (40%)	12 (33%)	Human skin / meningitis, nosocomial infections (skin/wound/ and pneumonia), meningitis, abscesses, bacteremia, urinary tract infection (UTIs), periodontal disease. Recurring oral ulcers (ROUs), wound infections, endocarditis, and osteomyelitis.
Chryseobacterium	0	6 (17%)	Soil and water / nosocomial, nosocomial mini-epidemics, neonatal meningitis, adult pneumonia, bacteremia, septicemia.
Pseudomonas	6 (40%)	0	Ubiquitous in moist environments / lower respiratory tract infections (RTIs), UTIs, eye infections, endocarditis, nosocomial infections, folliculitis, mengingitis, osteomyelitis, chronic lung infections in cystic fibrosis, wound and burn infections.

Acinetobacter, which are widely distributed in nature and are occasionally isolated from the human oral cavity, were found equally in the HMG and FMG. *Chryseobacterium*, whose natural habitat is soil and water, were found only in the FMG, while *Pseudomonas*, which prefers a moist environment, was found at a significantly ($p<0.001$) higher level in the HMG. There are major differences between the environment of the hockey arena and the environment of the football fields. FMGs have a much greater chance of contact with soil than HMGs. Hockey arenas and hockey lockers rooms tend

to be cooler and more humid than football fields and football locker rooms. Also, there are differences between the wearing patterns of mouthguards between hockey players and football players. Hockey players keep their mouthguards in their mouth for much longer periods of time than do football players. Football players tend to remove their mouthguards between plays, which allows the mouthguard to dry. These factors taken together suggest that generally the environment of the football mouthguard will be much different from that of the hockey mouthguard, this will result in different biofilms with different microbial populations forming on the two types of mouthguards.

An analysis of the distribution of *Staphylococcus* species is also interesting. (Table 15) *Staphylococcus spp.* is commonly found in human clinical specimens so it is not surprising that the majority of mouthguards were positive. *S. hominis,* which was isolated significantly more frequently ($p<0.001$) from FMG than HMG, is more commonly found on the skin in areas where apocrine glands are numerous [45]. It is not clear why *S. hominis* would be more common on football mouthguards, but it does suggest a difference in population between the two types of mouthguards. *S. capitis,* which has a preference for the areas of human skin where sebaceous glands are most numerous, [45] were also significantly ($p<0.01$) more common in the FMG than HMG. Again, the reasons for these differences are not apparent but suggest there are fundamental differences in the mouthguard/mouth environments that selects for or against specific species of bacteria.

Table 15. Distribution of *Staphylococcus* Species in Mouthguards
Number of mouthguards (%) positive for species

	HMG	FMG	Natural Habitat / Associated Diseases
Staphylococcus	11 (73%)	35 (97%)	The natural habitat of *Staphylococcus* in humans is the skin, skin glands and mucous membranes / normally symbiotic but pathogenic when they gain access deeper in host tissues.
			Coagulase-positive Staphylococcus
S. aureus	7 (47%)	11 (31%)	Preference for anterior nares/Major human pathogen bacteremia, pneumonia, osteomyelitis, acute endocarditis, myocarditis, pericarditis, cerebritis, meningitis, chorioamnionitis, Scalded Skin syndrome, Toxic Shock syndrome (TSS), Methicillin-Resistance Staph. aureus (MRSA)
			Coagulase-negative *Staphylococcus* (CoNS)
S. epidemidis	5 (33%)	21 (58%)	Major component of the human normal micro-flora / Leading cause of CoNS infections
S. hominis	1 (7%)	20 (55%)	Areas where apocrine glands are numerous (axillae and pubic area) / septicemia
S. capitis	0	10 (28%)	Areas where sebaceous glands are numerous especially scalp and forehead / septicemia, catheter infections, endocarditis
S. haemolyicus	1 (7%)	5 (14%)	Areas where apocrine glands are numerous (axillae and pubic area) / 2nd most common cause of CoNS, endocarditis, septicemia, peritonitis, urinary tract infections, wound, bone, and joint infections
S. xylosus	0	6 (17%)	Usually only transit on humans, primarily acquired from domestic animals / urinary tract infections

SELECTION OF YEAST BY ORAL APPLIANCES: Analyses of the yeast results strengthens the argument that the mouthguard/mouth environment is altering the oral microbial ecology of the mouthguard. *Candida* is the genus most commonly isolated from the oral cavity, and is found in approximately 50% of the normal healthy human oral cavities. Yeasts are not randomly distributed throughout the oral cavity but are localized in specific niches. The common sites for isolation of *C. albicans* from the oral cavity are the tongue and hard palate, (areas covered by keratinized stratified squamous epithelium). The method of sampling will have an affect on the isolation rate. (It must be noted that only 5/30 of the swabs (17%) from the medical students' oral cavity samples were positive for *C. albicans*. This low-level recovery was most likely due to the method of sampling). The cultures taken from the hockey players' mouthguards and used dentures were similar to direct contact cultures, and the results of 40% and 50% respectively positive for *Candida* supports this hypothesis. It is still unclear why only 3% of FMG, which were also similar to direct contact cultures, were positive for *Candida*. This is significantly ($p<0.01$) lower than would have been expected if selective effects were not at work.

C. albicans is the most common species of yeast isolated from the oral cavity. In normal healthy humans greater than 90% of the *Candida* isolates will be *C. albicans* [61]. The five *Candida* isolates from the MSI students were identified by the API database as *C. albicans./*. Even though the MS-I swabs yielded less than the expected number of yeast isolates, the fact that they were all identified as *C. albicans* supports the idea that it was the sampling method that resulted in the low yield.

In the used dentures, 50% of the Candida isolates were *C. albicans*. The other species of *Candida* isolated from the used dentures were one each of *C. lusitaniae*, *C. paratropicalis* and *C. sphaerica*. UD had the widest variety of yeast genera and species. Six of the UD yielded isolates of *Torulopsis glabrata*, yeast considered by many mycologists to be a species of *Candida*, *C. glabrata*. If *T. glabrata* is considered as a *Candida* then the number of UD positive for *Candida* would be 15/20, 75% positive for *Candida spp.* (one denture was positive for both *C. albicans* and *T. glabrata*). Of the UD positive for *Candida*; 47% were *C. albicans*, 40% *C. (T.) glabrata* and 7% other *Candida* species. These results are consistent with the research of Fidel et al. [21] who explained these results were due to differences in adhesive potential of denture plastic as compared to that of normal biosurfaces of the oral cavity. This example of microbial ecology alteration by oral appliances is inconclusive as to whether *C. glabrata* is replacing *C. albicans* or is moving into a separate niche. *Torulopsis (C.) glabrata* is increasingly recognized as an etiological agent of an emerging infectious disease. It occurs as a part of the normal flora of the oral cavity in a small percentage of the population. In the present study, *T. glabarata* was isolated only from used dentures. Six of the 20 UD yielded isolates of *T. glabarata*. This is a significant increase over what would have been expected if selection by the used dentures were not taking place ($p<0.001$). One important selection factor of the UD for *T. glabarata* is its relatively greater ability to adhere to the surface of methyl methacrylate dentures [51]. Effects of the increase in population of *T. glabrata* on the oral cavity microbial ecology and overall health of the host still need to be evaluated.

The yeast, *Rhodotorula*, is a normal soil inhabitant and is rarely isolated from the oral cavity. No *Rhodotorula* was isolated from the LR, UD, HMG or the medical students' oral swab samples. Samples taken from the practice and playing football fields yielded 6/40 (15%) isolates of *Rhodotorula*. *Rhodotorula* was isolated from 6/36 (17%) of the FMG. This is significantly higher ($p<0.001$) than would have been expected if the mouthguards were not picking up *Rhodotorula* from the soil. This strongly supports the idea that the source of *Rhodotorula* in the mouthguards was the soil.

Rhodotorula is normally not considered to be a pathogen. However, in the immune-compromised patient, especially when an indwelling catheter is in use, *Rhodotorula* can become a pathogen. In a review of English literature, Anatoliotabi et al. [3] discovered 47 cases of *Rhodotorula* fungemia in the period 1960-2001, with a majority being post 1990. The increased presence of *Rhodotorula* in the oral cavity is going to have an affect on the oral cavity microbial ecology. Whether the increased presence of *Rhodotorula* in the oral cavity also has an affect on oral health and general health is yet to be determined.

One strain of *Saccharomyces cerevisiae*, brewer's yeast, was isolated from the used dentures. *S. cerevisiae* is occasionally isolated from the oral cavity of normal healthy people. Brewer's yeast is usually considered to be non-pathogenic. However even something as innocuous as brewer's yeast may be a serious pathogen in the immune-compromised person [88].

The absence of *C. albicans* in the FMG is very interesting. The structure and chemical composition of football and hockey players' mouthguards are essentially the same, so the lack of *Candida* in the FMG is not due to differences in sampling or adhesive potential. One possible explanation might be the phenomenon of killer yeast. The total lack of *C. albicans* and the relatively high incidence of *Rhodotorula* 6/36 (17%) suggest this as a possibility.

The "Killer Yeast" phenomenon is similar to antibiotic production in bacteria. Killer capabilities can be induced in yeast by cytoplasmically inherited encapsulated ds-RNA virus, linear dsDNA plasmids or nuclear genes. The killer yeast secretes low molecular weight proteins or glycoproteins that are toxic to other yeasts. Many of these killer molecules function by specifically binding to cell surface structures on sensitive yeast. Following attachment, the killer protein inserts into the cell membrane and forms an ion pore allowing cell contents to leak out and thereby killing the yeast cell. Killer yeast are resistant to their own toxins. However, they may be sensitive to the toxins of other killer strains [52]. Yeast strains vary greatly in their sensitivity to killer yeast toxins [86]. Middlebeek et al. [60] found that many strains of *Candida* isolated from clinical specimens were highly sensitive to killer yeast toxins. Izgu et al. [41] found that some killer yeast toxins were also effective against some strains of gram-positive bacteria.

Many strains of *Rhodotorula* isolated from the environment show killer activity [85]. Whether the "Killer Yeast" phenomenon produces this lack of *C. albicans* in the FMG were not determined in this study. Due to the increased level of pathogenicity of

Candida, replacing *C. albicans* with the non-pathogenic yeast, *Rhodotorula*, does suggests some interesting clinical possibilities.

The other yeast isolates from the FMG, were all soil organisms and consisted of one isolates each of *C. lusitaniae*; *Cryptococcus spp.* and *Trichosporon spp.* This suggests that other "soil microorganisms" may also be "picked-up" from the soil by the FMG. These "soil microorganisms" would not be as likely to be found in HMG or UD. It is possible that some yet unidentified "soil microorganism" picked up by the FMG produce an anti-*C. albicans* factor. Whether it is a yeast killer factor or a bacterial antibiotic (or some intrinsic factor of the OSU football players' mouthguards), it seems to be very specifically effective against *C. albicans*. Whether this suppression of the *C. albicans* in the oral cavity is a positive or negative health factor is not clear. However, with the increased pathogenicity of *C. albicans* the identification of this factor might be very important clinically. What ever the cause, this significant reduction in *C. albicans*, is an example of mouthguards altering oral microbial ecology.

SELECTION OF *B. CEREUS* STRAINS BY MOUTHGUARDS: In this study, *B. cereus* was used as a "sentinel" organism, because of its ubiquitousness in the environment. Soil is a natural habitat of *B. cereus*, and is a common contaminant due to its ability to form resistant spores that move through out the environment. As a result food is commonly contaminated with *B. cereus*. Te Giffle et al. [83] in a study of pasteurized milk from household refrigerators in the Netherlands found 40% (133/334)

contained viable *B. cereus*. Although this is a study of only one food product from Europe, the situation is most likely very similar for other foods found in the USA. Consequently, we all have *B. cereus* routinely moving through our digestive systems.

The ubiquitous nature of *B. cereus* in dirt and dust is well demonstrated by this study. Soil samples were all positive for *B. cereus* spores. Therefore it is not surprising that anywhere dirt or dust is found, there is a good probability of finding *B. cereus*. By contrast, oral samples contain *B. cereus* at the rate of five percent or less, so the 25% positive rate for FMG is significantly higher ($P<0.001$) than expected. This strongly suggests football players' safety mouthguards pick up *B. cereus* from environmental dirt and dust. The close genetic relationship between members of the *B. cereus* group is a concern, since the close lineage within the *B. cereus* group includes *B. anthracis*, and there is always a strong possibility of the transfer of virulence factors within the group [37].

Te Giffle et al. [83] also found in his milk study that specific strains of *B. cereus* adapted to the environmental conditions of milk implying that, the environmental conditions of milk select for certain strains of *B. cereus*. Soil samples were taken from the SF, PF, and GF in a pattern that was intended to give a representative sample from the field. As the TPIs of the isolated *B. cereus* were correlated with the location on the field where they were isolated, there is the suggestion that environmental factors in different areas of the field are selecting for different strains. There also seemed to be selective differences

among the fields, so that only the PF, GF and LR were considered as the possible sources of *B. cereus* in the FMG.

In this study, *B. cereus* was isolated from the environmental sources and the mouthguards as spores. Twenty five percent of the FMG were positive for the *B. cereus* spores. These spores had been able to adhere to the mouthguards so that they were not removed by normal cleansing mechanisms. The adherence of the *B. cereus* spores was probably due to the relative hydrophobicity of the spores as compared to the hydrophobicity of the mouthguard. Ronner et al. [76] found that *Bacillus* spores adhere to a greater extent then the vegetative cells. The same 36 FMG that were cultured for *B. cereus* spores were also cultured by direct contact to look for aerobic bacteria in the vegetative cells stage (possibly also as spores). Nine of the 36 (25%) were positive for *B. cereus* by this method. However, only two of the total of 18 mouthguards positive for *B. cereus* were positive by both the spore culture and the contact culture methods.

Different strains of *B. cereus* have different hydrophobicities, which results in differences in their ability to adhere to the surface of a mouthguard or other surface within the oral cavity. In the vegetative cell this difference in hydrophobicity is due to the S-layer of the *B. cereus* cell. In *B. cereus* spores, the hydrophobicity is associated with the composition of the exosporium. Because only two out of eighteen were positive by both methods, it appears that the spores and vegetative attached by different mechanisms. This suggests that there is a different selective factor at work between mouthguards and vegetative cells of *B. cereus*. This implies that the FMG or perhaps the biofilm formed

on the FMG have a different selective effect relative to the hydrophobicity of the vegetative *B. cereus* cell.

The finding of *B. cereus* spores in one group of mouthguards and vegetative cells in a second group of mouthguards raises a multitude of questions. The original assumption was that if the spores adhered they would germinate and become vegetative cells. However, the question now is whether the spores that attach are not able to germinate, or perhaps the spores attaches and germinates but then the vegetative cells are then removed by the normal processes, or perhaps a different process entirely is in operation. The results of this study did not provide a clear-cut answer. One of the questions of this study was to determine if the mouthguard/mouth select for specific strains of *B. cereus* spores from the reservoir of *B. cereus* spores in the football players' environment. The answer to that question is definitely "yes". But the results also suggest that there are more complex interactions taking place between the mouthguard/mouth environment and the selection of *B. cereus* strains.

The results of this study indicates that selection with reference to toxin production of *B. cereus* strains was taking place in the football players' mouth/mouthguard. The selection factor involved is probably the hydrophobicity of the spores relative to the hydrophobicity of the mouthguards. This suggests that there is a relationship between hydrophobicity of *B. cereus* spores and exotoxin production of the daughter vegetative cells. The nature of this interesting relationship needs further exploration.

B. cereus is usually considered to be non-pathogenic, but this study categorized *B. cereus* by profiling toxin production. Specifically, although five different *B. cereus*'s toxins were tested, this is not an exhaustive list of *B. cereus* toxins. There are cooperative and synergistic effects among the various exotoxins to produce the observable effects. To add to the complexity, these effects are also moderated by environmental factors. Two identification criteria of *B. cereus* are its ability to hemolyze sheep red blood cells and to break down egg yolk lecithin. Both the effects of hemolysis and lecithinase are produced by one or more exoproteins. These capabilities are effects of exoproteins produced and secreted by all *B. cereus* when grown on sufficiently complex medium. The production of these exoproteins has an energy cost to the bacterial cell, so are mainly produced in reaction to a stimulus in the environment (media). When a strain of *B. cereus* is grown on a simple media containing fewer possible stimulants, the spectrum and amounts of exoproteins change, and the effects may be suppressed.

The association between *B. cereus* strains in the oral cavity and specific health problems is yet to be determined. This study shows that the mouthguards can selectively "pick-up" *B. cereus* spores from the environment. The spores are metabolically inactive, but they are foreign proteins to the human host. The vegetative cell produces a whole host of exoproteins, many of which are human toxins. While the effects of some toxins are obvious, such as enterotoxin and emetic toxin, others such as the effect of the emetic toxin on human natural killer cells is much harder to document. These results suggest that the aspiration of "non-pathogenic" *B. cereus*, with its spectrum of exotoxins, could possibly have a significant, as yet unrecognized, effect on the health of the host. Also,

the *B. cereus* spores, vegetative cells, and cellular products in close proximity to host cells, will induce an immune response in the human host.

The key as to whether *B. cereus, Rhodotorula*, C. *(Torulopsis) glabrata* or some as yet unidentified microorganism is pathogenic or non-pathogenic in the human oral cavity most likely depends on the organism's interaction with the oral cavity. The first requirement of pathogenesis is that the microorganism must be able to adhere to the oral cavity. This study has shown that the wearing of an athletic mouthguard can provide sites and conditions that allow microorganisms not routinely isolated from the oral cavity to adhere to surfaces on the mouthguard. The question now is "Will the presence of these microorganisms invoke an immune response in the host and what will be the effect of this immune response?"

HUMAN IMMUNE SYSTEM VS. ORAL CAVITY MICROORGANISMS: Rossi et al. [77] in a related study measured salivary cytokine levels of football players at different times during a college football season. The FMG for culturing were collected following a game during this same football season. The results of the Rossi study suggest that over the course of a competitive college football season, the salivary cytokine levels of TNF-α and INF-γ increase. It is very difficult to correlate changes in microorganisms with changes in cytokines levels. One reason for this is that the Major Histocompatability Complex (MHC) programs the oral cavity immune response. The MHC is based on multiple genes and is probably as individualized as fingerprints, so

there can be major differences between individual human host's immune response to the same microorganism.

Also, many studies have shown that intensive exercise, with or without athletic safety mouthguards, can alter the oral cavity immune response. Intensive exercise can specifically result in decreased levels of sIgA in saliva to the extent that the individual is at risk of upper respiratory tract infection [31]. Given the importance of sIgA in the oral cavity immunity, lowering the level of sIgA will have a significant effect on oral cavity ecology. This lowered level of sIgA may even be a factor in allowing "foreign" microorganisms to be incorporated into the mouthguard/mouth oral cavity microbial ecology. Whether or not the intensive training of college football players will lower their mucosal immunity to the extent that *Rhodotorula* and select strains of *B. cereus* are able to colonize the mouthguard biofilm is a matter of conjecture. It would be of interest to determine if there is a relationship between such things as sIgA level and distribution of *Staphylococcus* species in the oral cavity.

Steel and Fidel [81] studied the secretion *in vitro* of cytokines, including TNF-α and IL-1a, from human oral epithelial cells in response to exposure to *C. albicans*. It would be very interesting to use similar methods to evaluate and compare different species of non-pathogenic soil microorganisms including yeast and bacteria, and specific strains of *B. cereus* isolated from FMG for their ability to induce cytokine production from oral epithelial cells.

SUMMARY: This study has shown that wearing an athletic protective mouthguards can alter the normal ecology of the mouth. Analysis of the incidence of some of the bacteria usually considered being part of the normal flora of the oral cavity shows some alterations in populations of these microorganisms. The study of oral cavity yeast reveals alterations in incidence of different genera, and the introduction of the yeast genera, *Rhodotorula.* Furthermore, this study shows that in the football players' mouthguards the incidence of the gram-positive bacteria, *B. cereus,* is significantly increased. Not only is the incidence of *B. cereus* increased, but also the mouthguard/mouth environment selects for specific strains of the bacteria. These findings suggest that the mouthguard/mouth environment may select other types of bacteria, fungi, virus or amoebae that may be more pathogenic then these "sentinel" organism. These new members of the oral cavity microbial community and their cellular products will induce alterations in cytokine production by the host's cells, which will subsequently alter the host's immune response. Because of these alterations, the wearing of athletic safety mouthguards may not be as benign as they are usually assumed to be. The affects on the oral cavity ecology of the protective mouthguards, which currently receives little attention, may have real significance in the health of the athlete wearer.

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APPENDIX A – Newspaper article – ***Clarmore Progress*** 24 October 2002

STATE ▪ OBITUARIES ▪ WEATHER

Too much exercise a bad thing?

Mouthpieces tested for germs

By DOTTIE WITTER
For CNHI News Service

STILLWATER — Student and professional athletes may start paying more attention to their mouthpieces in the future thanks to research being done by Dr. Melody Phillips.

Apparently "clean" mouthpieces harbor germs that could contribute to increased infection and injury, especially as the season progresses.

"Even when you chew, you have microscopic tears in your gums that act as a direct portal of entry for anything growing on the mouthpiece," Phillips said. "Changing or disinfecting mouthpieces daily may be very important to the health of athletes."

Phillips, an exercise physiologist in the Health and Human Performance Program in Oklahoma State University's College of Education, along with her colleagues Drs. Robert Conrad and Tom Glass at OSU's Center for Health Sciences, have collected mouthpieces that are now being cultured to determine what might be growing on them.

"We hope to access any potential immune response that may be consequent to the wearing of those mouthpieces all season," she said.

Her major research interest is the influence of exercise on the immune system and how those changes may affect one's health. She hopes to learn both the positive and negative influences of exercise, especially how to maximize the positive and minimize the negative.

"Exercise science research benefits the average person wishing to improve health, as well as student or Olympic athletes who need to minimize injuries and improve performance," she said.

While exercise is known to improve health, overtraining is harmful, she said. "This is something that got me interested in immunology. Why would they get sick more often?"

Research has shown that as weekly training volume increases above an optimal point, one's risk of infection increases.

"Researchers think that's because of something known as 'the open window hypothesis,' which describes a period of time after a hard or very long exercise bout during which the body may be more susceptible to infection," she said.

"If athletes exercise hard enough and long enough, it might actually suppress the immune system."

To learn more about exercise and the immune system, Phillips and her research team is studying cytokines, the inflammatory proteins in the blood that are also associated

with cardiovascular disease, osteoporosis and diabetes.

Think of cutting your finger. The tissue around the cut becomes red, painful and inflamed. The inflammatory cytokines stimulate this immune response. Fever also is regulated partially by cytokines.

Cytokines are also produced by your bone cells, and when certain conditions exist, like menopause, the bone degrading cells become more active.

"Bone degrading cells are naturally active in our body, but if they are over-stimulated, then people will experience bone loss," she said. Cytokines stimulate these bone-degrading cells.

Phillips' previous research found that just 10 weeks of resistance training for elderly women decreased the production of the inflammatory cytokines in whole blood.

The influence of exercise, specifically exercise training on cytokines in elderly and at risk populations, might be of great benefit to another growing segment of the population.

"Oklahoma has a large population at risk for Type II Diabetes, the adult-onset diabetes," Phillips said, citing the large American Indian population.

The cytokine most associated with insulin resistance in diabetes decreased significantly after 10 weeks of moderate to intense training, Phillips said.

"I would like to continue my line of research, to investigate the influence of resistance training in populations predisposed to having diabetes, and look at the relationship of cytokines and insulin sensitivity."

Dottie Witter writes for the OSU News Service

PHOTO PROVIDED

Dr. Melody Phillips (far left) helps gather players' mouthpieces to test for germs.

"If athletes exercise hard enough and long enough, it might actually suppress the immune system."

Obituary

Patrick Allison O'Bannon

Services for native Claremore businessman, Patrick Allison O'Bannon will be 10 a.m. Friday, Oct. 25, 2002 at First Presbyterian Church. Rev. Dennis Ritchie will officiate. Burial will follow at Woodlawn Cemetery, under the direction of Rice Funeral Service. Friends may visit at the funeral home until 8 p.m. Thursday.

Pat passed away Wednesday morning, Oct. 23, 2002. He was 79.

Born in Claremore on July 19, 1923 to Frank and Maurine O'Bannon, Pat was reared and educated in Claremore, graduating from Claremore High School in 1941. He played football and was chosen for the All-State Football Team. He graduated from OU with a bachelor's degree in business and then served his country in the Air Force, in the 3rd Emergency Rescue Squadron in the South Pacific during World War II. Following his honorable discharge, Pat went to work selling oil field equipment as manger of Pacific Pumps and made his home in Texas. It was here that he met and married Francis Mae Copeland and the two settled in Odessa. The couple was blessed with one son, David.

Thirty-six years ago, the O'Bannon family returned to Claremore and Pat owned and operated the Claremore Credit Bureau until its sale, after which he immediately opened Allison Loan and Realty. Pat was very involved in the community, giving of his time and means. He was a great believer in education and supported Rogers State University and Oklahoma University. He was a 32-year member of Claremore Rotary, and a member of the American Legion and Elks Lodge. Proud of his Irish ancestry, he was a founder of the annual Claremore St. Patrick's Day Parade. Several times in recent years, Pat led the parade as grand marshal. He was a member of the First Presbyterian Church.

Pat's early love of football continued throughout his life. He cheered for OU and the Dallas Cowboys. He enjoyed traveling whenever the opportunity arose and fishing was another favorite activity. His daily walks were a source of enjoyment.

Pat was a generous, helpful man and a loving and supportive father. He is survived by his wife of 39 years, Francis of Chelsea; son, David O'Bannon of Tulsa and David's fiancee, Kathy Olsen of Pryor. He was preceded by his parents and brother, Michael O'Bannon.

DOC officially announces delays in furloughs

OKLAHOMA CITY (AP) — The Oklahoma Department of Corrections officially announced Wednesday that it would postpone furloughing 4,850 employees for a month.

DOC officials had scheduled days off without pay for the employees to begin Nov. 1. Instead, the furloughs will begin Dec. 1.

Senate President Pro Tem-elect Cal Hobson said Tuesday that a pact had been obtained to postpone the furloughs.

Director Ron Ward said the delay will give employees more money for the holiday season and give lawmakers more time to devote to solving the department's fiscal crisis.

"A lot of us felt this was an opportunity to stand back and see what approaches we could take to offset these furlough days," Ward said.

The department, like other state agencies, is having to cut its budget because of decreased tax revenue.

The DOC has to cut $18.6 million from its budget. The agency already had faced a projected $12 million deficit before cutbacks were ordered.

The $30.6 million funding shortfall has been reduced to $29 million through reducing costs, minimizing spending and enacting a hiring freeze, Ward said.

Furloughs for 23 days, the maximum allowed by state law, will save the department $14.9 million, Ward said. November's planned furlough days will be moved to later in the fiscal year, department spokesman Jerry Massie said.

By delaying the furloughs, corrections employees will receive a full paycheck before the Christmas holiday.

House Speaker Larry Adair said the extra month gives everyone time to try to resolve the furlough issue.

"I think everyone was concerned about the employees who would be affected," Adair, D-Stilwell, said. "Even though we weren't able to completely resolve the issue, it gives us some time to continue working."

Corrections Board member Beverly Young said the department's employees are suffering from the poor decisions that have been made about how Oklahoma incarcerates its criminals.

"We have a lot of people incarcerated for nonviolent offenses," Young said. "We need to figure out who we're mad at and who we're afraid of. It's not out-of-sight, out-of-mind anymore. We can't afford it."

The Oklahoma Public Employees Association also is seeking longer-term solutions to the department's funding woes.

Dean Balmer, the association's coordinator for corrections issues, said he has a proposal to save the department $8 million.

APPENDIX B – INSTITUTIONAL REVIEW BOARD APPROVAL

Oklahoma State University
College of Osteopathic Medicine

Institutional Review Board

Memo

To: R. Tom Glass, D.D.S., Ph.D., Principle Investigator
Department of Pathology

From: Dianne Miller-Hardy, Ph.D., J.D.
Chairman, Institutional Review Board

Date: January 6, 2000

CC: Robert S. Conrad, Ph.D., Co-Investigator
Ortwin Schmidt, Ph.D., Co-Investigator

Re: ***In vivo* Evaluation of Denture Decontamination Using the MicroDent Sanitizing and Cleansing System**

This protocol was reviewed and approved during the January 5, 2000 Institutional Review Board meeting. You are now free to begin your research. If you have any questions, please contact me at extension 8406.

APPENDIX B – INSTITUTIONAL REVIEW BOARD APPROVAL – 2002003

Oklahoma State University
Center for Health Sciences
College of Osteopathic Medicine
Institutional Review Board

Memo

To: R. Tom Glass, D.D.S., Ph.D.
Professor of Pathology

From: Stephen Eddy, D.O., M.P.H. 7/2/03
Chairman, Institutional Review Board

Date: July 2, 2003

Re: **Accept Continuing Review**
Approve Continued Study
Protocol - IRB # 2002003

Title: **Evaluation of the Microbiota in Athletic Mouthpieces and the Reactive Immune Constituents in the Saliva of Football Players from Oklahoma State University and a Group of Medical Students from Oklahoma State University Center for Health Sciences**

Your request for continuing review of the study listed above was reviewed at the July 2003 meeting of the OSU-CHS Institutional Review Board.

The requested continuation involves no changes to the protocol. This is to confirm that your request for continuation is approved. The study is approved through July 2004.

You are granted permission to continue your study as described effective immediately. The study is next subject to continuing review on or before July 2, 2004 unless closed before that date.

If you have any questions, call Teri Bycroft, IRB Administrator, at (918) 699-8643.

APPENDIX B – INSTITUTIONAL REVIEW BOARD APPROVAL – 2002003

Oklahoma State University
Center for Health Sciences
College of Osteopathic Medicine
Institutional Review Board

Memo

To: R. Tom Glass, D.D.S., Ph.D.
Professor of Pathology

From: Stephen Eddy, D.O., M.P.H. 1/21/04
Chairman, Institutional Review Board

Date: January 21, 2004

Re: **Accept Continuing Review**
Approve Continued Study
Protocol - IRB # 2002003

Title: **Evaluation of Mouthpiece Microbial Contamination *In vivo***

Your request for continuing review of the study listed above was reviewed at the January 2004 meeting of the OSU-CHS Institutional Review Board.

The requested continuation involves no changes to the protocol. This is to confirm that your request for continuation is approved. The study is approved through January 2004.

You are granted permission to continue your study as described effective immediately. The study is next subject to continuing review on or before January 21, 2004 unless closed before that date.

If you have any questions, call Teri Bycroft, IRB Administrator, at (918) 699-8643.

VITA

JAMES W. BULLARD

July 2004

PERSONAL HISTORY

College Address:
Dept. of Biochemistry/Microbiology
College of Osteopathic Medicine
Tulsa, Oklahoma 74107-1898
(918) 561-8492

Home Address:
3710 South Redbud Drive
Sand Springs, Oklahoma 74063
(918) 241-6890

Birth date: 21 October 1943

Martial Status: Married (4 grand children)

EDUCATIONAL HISTORY

1. University of Tulsa
   Tulsa, Oklahoma
   Legal Assistant Certificate Program
   Completed 1995

2. Water and Wastewater Treatment School
   Neosho, Missouri
   Water and Wastewater Treatment Technology Certificate Program
   Completed 1975

3. Menorah Medical Center
   Kansas City, Missouri
   Medical Technology (American Society of Clinical Pathologist ASCP)
   Completed 1970

4. University of La Verne
   La Verne, California
   Major: Environmental Management
   Degree: Bachelor of Science 1980

5. Southwest Missouri State University
   Springfield, Missouri
   Major: Biology
   Degree: Bachelor of Arts 1966

6. Oklahoma State University – Center for Health Sciences
   Tulsa, Oklahoma
   Completed requirements for Masters of Science degree July 2004

PROFESSIONAL POSITIONS

1. Senior Research Assistant
   Oklahoma State University – Center for Health Sciences
   Tulsa, Oklahoma
   September 1997 – Present

2. Medical Technologist III (Immunology)
   Regional Medical Lab – St. John Medical Center
   Tulsa, Oklahoma
   1994-1997

3. Chief Medical Technologist / Lab Manager
   Immuno-Diagnostics Laboratory
   Tulsa, Oklahoma
   1982-1994

4. Veterinarian Medical Technologist / Research Technologist
   Oral Roberts University
   Tulsa, Oklahoma
   1980-1982

5. Resident Director
   Central Texas College, Subic Bay Naval Station Campus
   Republic of the Philippines
   1979-1980

6. Assistant Area Director / Resident Coordinator
   Los Angeles Community Colleges Overseas, Los Angeles, California
   Subic Bay Naval Station Campus
   Republic of the Philippines
   1978-1979

7. Lead Instructor / Administrator
   Water and Wastewater Technical School, Neosho, Missouri
   Clark Air Force Base Campus
   Republic of the Philippines
   1976-1978

8. Operations Specialist
   Environmental Laboratories Inc., Topeka Kansas
   1975-1976

9. Medical Technologist / Missionary
   Central Hospital of Il Maten, Algeria
   United Methodist Church Board of Global Concerns, New York, New York
   1970-1974

10. Medical Technologist
    Sale Memorial Hospital, Neosho, Missouri
    1970-1971

11. Quality Control – Method Development Chemist
    Anchor Serum Division of Philips Roxanne Corporation, St. Joseph, Missouri
    1968-1969

12. Medical Lab Specialist
    U.S. Army, Frankfurt, Germany
    1967-1968

REGISTERIES

Registered Specialist in Immunology **SI** (ASCP)

Registered Medical Technologist **MT** (ASCP)