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A STUDY OF WATER-BORNE BACTERIAL PATHOGENS IN THE CONTEXT OF THE DENTAL OPERATORY

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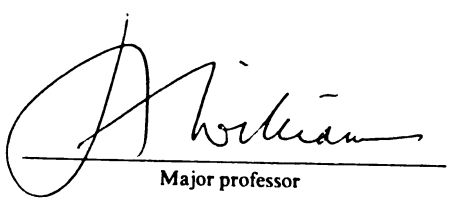

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A STUDY OF WATER-BORNE BACTERIAL PATHOGENS IN THE CONTEXT OF THE DENTAL OPERATORY

by

Mark Kenneth Huntington

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ABSTRACT

A STUDY OF WATER-BORNE BACTERIAL PATHOGENS IN THE CONTEXT OF THE DENTAL OPERATORY

by

Mark Kenneth Huntington

Reports of microbiologically contaminated dental water come from all corners of the globe. As early as 1963, significant pathogens were isolated from dental water systems. In light of increased attention to infection control in recent years, my studies reported herein were undertaken to evaluate and characterize the microbiological quality of water in the modern dental operatory. Culture of dental unit water revealed enormous contamination by bacteria, including a variety of well known pathogens and saprophytic organisms, identified by their biochemical profiles. Bacterial concentrations in the dental water samples were exponentially greater than those of potable water systems. The proximate source of this contamination is the biofilms lining the small caliber instrument cooling and irrigation water lines.

Gram negative bacteria were the most prevalent organisms in the dental water samples. Correspondingly high

concentrations of endotoxin were measured in the water using the *Limulus* amoebocyte lysate assay. Endotoxin concentration correlated positively to the bacteria load present. Aerosolization of endotoxin during dental procedures was detected using an aerosol-sampling impinger.

Also prominent among the contaminants was *Legionella*. Surveys utilizing the EnviroAmp^{tm} polymerase chain reaction (PCR) detection system found *Legionella* present in two-thirds of the dental unit water samples. This prevalence rate was similar to that found in potable water samples collected over the same period. However, semiquantitation of the levels revealed a vastly greater intensity of contamination in the dental samples. The feasibility of using molecular fingerprinting, repetitive element PCR (rep-PCR), and direct fluorescent antibodies to study the ecology and epidemiology of dental water *Legionella* is demonstrated. Technical shortcomings were encountered with the use of the EnviroAmp^{tm} kit due to inhibitory factors present in the heavily contaminated dental water. The implications of this limitation to the technique are discussed.

These investigations reveal that serious contamination of dental unit water continues to be widespread and cannot be

ignored in the context of responsible infection control in the dental office.

An appendix describing the preliminary characterization of bombesin-like peptides and receptors in nematodes is attached.

To Charlene, for everything...

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Special gratitude is reserved for my family: my parents who, though lacking formal higher education themselves, have encouraged my pursuits, and my dear wife and children who have lovingly and supportively accompanied me on this long and often rough journey. Thanks to God.

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INTRODUCTION

Modern dentistry has significantly improved the overall health of the industrialized world. The incidence of dental caries, formerly one of the most common human afflictions, has declined significantly in recent years. Today, fewer than half of those under 17 years of age in the United States have carious lesions. Even periodontal disease, responsible for more lost teeth than caries, has shown declining prevalence in recent years [Greenspan 1991].

Routine dental care is, however, not a benign procedure. As with all medical interventions, certain risks are inherent. Some of these risks have been common knowledge for many years, others are only now beginning to receive the recognition they deserve. The research presented in this dissertation addresses aspects of the infectious complications of routine dental care, specifically,

contamination of dental water systems by heterotrophic organisms, *Legionella*, and microbial-derived endotoxin. The significance of these contaminants in the dental setting to public health has not been fully appreciated in the past and has only recently become the focus of increasing attention, as evidence has accumulated of infections acquired by patients, and of the occupational exposure of dental personnel to waterborne pathogens and opportunists.

This literature review includes discussion of: dental operatory design; dental water microbial contamination, including its characteristics, origins, and efforts at control; the medical significance of heterotrophic bacteria, *Legionella*, and endotoxin; and other documented infectious complications of dental care. The discussion provides the foundation for the original research presented in subsequent chapters of the dissertation on characterization of dental water contaminants and their products, and the detection and characterization of *Legionella* species.

THE DENTAL OPERATORY

The modern dental office is the product of years of continued improvement in ergonomic and efficiency

engineering. At its heart is the operatory. Over the years it has evolved from an armature for a low-speed drill (that literally burned its way through the enamel) with a small porcelain emesis basin, to a sophisticated microprocessor controlled center for a myriad of state-of-the-art instruments and a high efficiency water distribution system.

The dental water system is vital to the functioning of the operatory. Water enters the unit from either the building plumbing or a bottled water reservoir. The water is often heated to 35C for patient comfort. Some manufacturers add low levels of biocides or expose the water to ultraviolet irradiation in an effort at bacterial contamination control. The water is then diverted through a series of fine-caliber tubings and valves to serve as coolant for the high-speed handpieces ("drills" to the uninitiated), coolant and conduction medium for ultrasonic scalers (which remove tartar without scraping), and irrigant for the procedure site via the air-water syringe (the air being used in conjunction with the water to keep the procedure site clear of debris). Incorporated into the dental water distribution system are one or more anti-retraction valves designed to reduce "suckback" of patient material from the distal terminal of the line upon cessation of water flow to the instrument.

Replacing the cuspidor (spitoon) in the modern operatory is a suction system known as the saliva ejector. It consists of a disposable cannula which is placed in the patient's mouth and connected to a vacuum line. This device is designed to eliminate the need of the patient to "lean over and spit", increasing the efficiency of the dental procedure. A schematic diagram representative of the typical modern dental operatory is shown in Figure. 1.

DENTAL WATER CONTAMINATION

Reports of microbiologically contaminated dental water come from all corners of the globe, including the Americas, Europe and Scandinavia, Asia and the Pacific [Fitzgibbon *et al* 1984, Furuhashi *et al* 1985, Kelstrup *et al* 1977, Tamazawa *et al* 1992, Tippett *et al* 1988]. As early as 1963, organisms of significance to human health were isolated from dental water systems [Blake *et al* 1963].

The levels of contamination found are disturbing. Whereas municipal water supplies deliver negligible amounts of viable bacteria, dental operatories routinely deliver 10^3-10^7 organisms per milliliter [Abel *et al* 1971, Clark *et al*

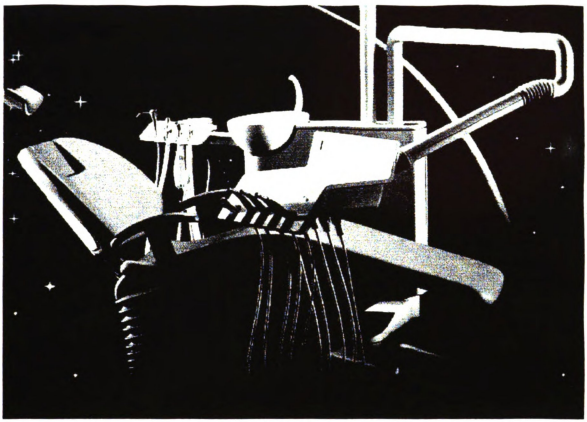


FIGURE 1. Photograph of a modern dental operatory. Note the long runs of tubing. Photo courtesy of PELTON & CRANE.

1974, Fitzgibbon *et al* 1984, Holbrook *et al* 1978, Kelstrup *et al* 1977, Larato *et al* 1966, Lewis *et al* 1992, Mayo *et al* 1990, Oppenheim *et al* 1987, Pankhurst *et al* 1990, Pankhurst *et al* 1993, Scheid *et al* 1982, Williams *et al* 1993]. In discussing his findings, Abel cited a 1923 United States Department of Treasury ruling that "water that produces counts greater than 100 (organisms per milliliter) is unfit to be served by common carriers in interstate traffic." The U.S. Army Medical School has set arbitrary limits of 500

bacteria per milliliter for potable water. Abel concluded that "all of the dental unit water supplies in this study would be considered contaminated and unfit for human use [Abel *et al* 1971]." Even in the sterilization-conscious 1990s, similar concentrations of bacteria continue to be present in dental water lines [Mayo *et al* 1990, Santiago *et al* 1994].

CHARACTERISTICS OF THE CONTAMINATION

The earlier papers dealing with dental water contamination were concerned with oral microbes and the risks associated with their aerosolization [Holbrook *et al* 1978, Micik *et al* 1969, Miller *et al* 1971]. Following reports that dental water bacteria were primarily saprophytic rather than pathogenic, the focus of concern became more aesthetic, based on the inappropriateness of administering large numbers of "harmless" slime-forming bacteria to dental patients and onto oral lesions [Abel *et al* 1971, Clark *et al* 1974, Fitzgibbon *et al* 1984, McEntegart *et al* 1973]. Again, contemporary thought regarding water "saprophytes" has changed and currently emphasizes their potential role in human diseases [Pankhurst *et al* 1993, Williams *et al* 1993, Williams *et al* 1994].

Published reports may be compiled to provide a long "laundry list" of organisms found contaminating dental water systems. Perhaps the most significant components are the bacterial populations. While no extensive speciation of these organisms was reported prior to the work presented in this dissertation, many papers include small-scale efforts at identification of the organisms present. Gram negative "saprophytes" such as *Caulobacter* and *Flavobacterium*; gram negative and gram positive pathogens such as *Pseudomonas, Alcaligenes, Moraxella, Proteus, Klebsiella, Bacteroides, Pasteurella, Acinetobacter, Neisseria,* staphylococci, streptococci, and enterococci; and other organisms such as *Legionella* and *Mycobacterium* have been isolated and identified [Abel *et al* 1971, Clark *et al* 1974, Fitzgibbon *et al* 1984, Holbrook *et al* 1978, Kelstrup *et al* 1977, Larato *et al* 1966, Lewis *et al* 1992, Mayo *et al* 1990, Oppenheim *et al* 1987, Pankhurst *et al* 1990, Pankhurst *et al* 1993, Scheid *et al* 1982, Williams *et al* 1993].

In addition to the prokaryotic component, eukaryotic organisms have been isolated. These include protozoa such as *Acanthamoeba* and *Naegleria* [Michel *et al* 1989, Williams *et al* 1993], a variety of fungi [Williams *et al* 1993], and nematodes [Michel *et al* 1989, Santiago *et al* 1994]. *Naegleria* and *Acanthamoeba*, known to cause amoebic

encephalitis following infection of nasal mucosa via exposure to contaminated water [Markell *et al* 1986], presents a risk directly to patients via the aerosols generated in the dental operatory, and indirectly, as in the case of *Acanthamoebae*, by serving as host for *Legionella* multiplication within the waterline.

While viruses have not been identified in dental water, there is no record of anyone having looked for them. Obviously, they do not proliferate extracellularly in water; nevertheless, the detection of human-derived bacteria in the line suggests the possibility for cross infection by viral organisms to occur via the waterline. Human viral nucleic acid sequences have been found in dental water in experimental situations, and intact bacteriophages enter the waterline when handpieces are operated with the tip in a phage suspension [Lewis *et al* 1992].

Hepatitis transmission (both hepatitis B and nonA-/nonB-viruses) has been well documented in the context of the dental operatory [Ahtone *et al* 1983, Fischer *et al* 1986, Mori *et al* 1984, Porter *et al* 1990, Schiff *et al* 1986, Tzukert *et al* 1978]. In addition to needle-stick accidents, mucous membrane contact, and nonsterile instrument cross-infection modes of transmission common to any medical

setting; suckback of blood, tooth particles, tissue fragments, and saliva into the dental water system occurs [American Dental Association 1988, Bagga *et al* 1984, Crawford *et al* 1988, Crawford *et al* 1990, Hadler *et al* 1981, Levin *et al* 1974, Lewis *et al* 1991, Lewis *et al* 1992, Manzella *et al* 1984, Miller *et al* 1985, Miller *et al* 1993, Reingold *et al* 1982, Shaw *et al* 1986]. These contaminants may ultimately be discharged into subsequent patients' mouths to be aspirated, ingested, or directly inoculated into their bloodstream.

Other viruses may be transmitted similarly. In a highly publicized case, five patients of an HIV-positive dentist were shown to be infected by the same strain of virus affecting their provider [Ou *et al* 1992]. While the exact mode of transmission of the virus remains open to speculation [Centers for Disease Control and Prevention 1991, Ciesielski *et al* 1992], needlestick injury, cross-infection by unsterile equipment, and dental water system contamination have all been suggested [Anonymous 1992]. Cytomegalovirus, herpes simplex virus type 1, herpes simplex type 2, and other viruses, specifically those that infect the upper respiratory tract, are also linked to dental care [Centers for Disease Control and Prevention 1993, Manzella *et al* 1984]. It is clear from the literature that dental

professionals and dental students suffer upper respiratory tract infections, common colds, and other respiratory ailments disproportionately compared to the general population [Burton *et al* 1963, Carter *et al* 1953, Mandel *et al* 1993]. The likely source of these infections is the daily exposure to aerosols in the dental operatory. While obviously of lower mortality than HIV or HBV, these infections do represent serious causes of morbidity.

ORIGINS OF THE CONTAMINATION

Within the waterlines a complex biofilm forms (Figure 2), originating with organisms derived from the municipal water supply. The low numbers of bacteria present in the municipal water supply (generally fewer than 50 organisms per milliliter [Abel *et al* 1971, Mayo *et al* 1990]) adhere to the small-bore plastic dental lines, changing from a planktonic to a sessile stage. A symbiotic relationship occurs between different organisms, with some producing a glycocalyx slime matrix (known as extracellular polymeric substance, or EPS) which serves as an anchor for both themselves and other organisms. Both environmentally derived organisms and organisms from patient's oral microbiota that gain access to the lines as the result of

the inadequate antiretraction mechanisms found in most dental water systems may participate in the adherent microbial consortia [Williams *et al* 1996]. The EPS serves as an anion exchange resin, capable of trapping nutrients based on their ionic charge, and preventing many biocides from reaching the resident organisms.

As the biofilm matures, various microenvironments are generated as a result of the nutrient and oxygen gradients that develop within the EPS. The result is that mature biofilms are complex communities with aerobic, microaerophilic, and anaerobic bacteria, filamentous fungi, a variety of protozoa (most notably amoebae), and even eukaryotic organisms (e.g. nematodes such as *Rhabditis*) each finding a niche in which they can thrive [Characklis *et al* 1983, Costerton *et al* 1978, Costerton *et al* 1981, Costerton *et al* 1984, Costerton *et al* 1987, Marshall *et al* 1992, Mayette *et al* 1992, Santiago *et al* 1994].

Biofilms are significant to the medical community for other reasons. They form at many fluid-solid interfaces and are associated with pacemakers, prosthetic joints, other medicaldevices, and prolonged use of implanted catheters. Biofilms are implicated in the development of urinary tract

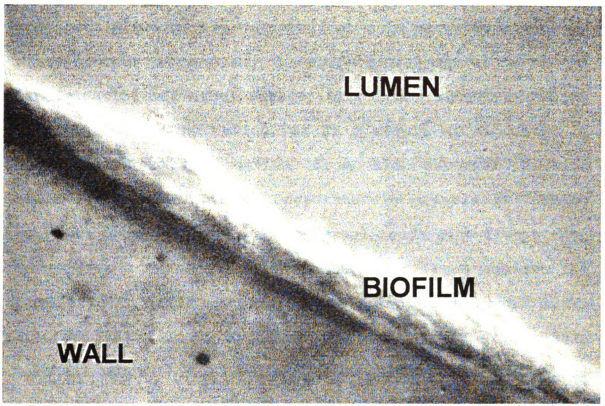


FIGURE 2. Biofilm in a dental waterline. This cross-section of a dental waterline shows the lush growth of biofilm lining the lumenal surface of the tubing (enlarged approximately 1200X). Reprinted by permission of ADA Publishing Co., Inc. [Williams *et al* 1993].

infections, endocarditis, catheter-related sepsis, local abscesses, necrosis, implant rejection, and cellulitis.

Often, biofilm contamination of the surface of prostheses necessitates their removal and replacement [Costerton *et al* 1987, Gristina *et al* 1984, Gristina *et al* 1988, Kluge *et al* 1982, Marrie *et al* 1982, Nickel *et al* 1992, Peters *et al* 1981, Russell *et al* 1987].

Most such biofilms are derived from skin microbiota such as *Staphlococcus aureus* and *S. epidermidis*, as a result of contamination of the device prior to surgery, during implantation of the device, or as a result of migration of organisms down the device from its external portion following placement. The organisms are capable of forming biofilms along catheters and external leads with fronts that advance at a rate of over 2 cm per hour, even against the flow of antibiotic containing fluids [Anwar *et al* 1992, Gristina *et al* 1988, Kluge *et al* 1982, Marrie *et al* 1982, Marshall *et al* 1992, Nickel *et al* 1992, Passerini *et al* 1992, Peters *et al* 1981]. Biofilm organisms may also be spread hematogenously during episodes of bacteremia (even asymptomatic transient bacteremias). Dental procedures result in such bacteremias. Controlled experiments involving otherwise healthy rabbits confirmed that bacteremia follows oral manipulations in a significant number of animals [McGowan *et al* 1994]. These bacteremias have been implicated in infections of artificial joints [Grant *et al* 1992, Little *et al* 1991, Thyne *et al* 1991], artificial heart valves and pacemakers [Pavek 1990], intracranial shunts [Thyne *et al* 1991], and penile implants [Drinnan *et al* 1990, Little *et al* 1992].

Every first-year medical student learns of the well established correlation between subacute bacterial endocarditis and dental care. This condition occurs when a transient bacteremia of alpha-hemolytic streptococci results in the formation of a biofilm on heart valves, most often in the presence of a congenital or acquired (e.g. rheumatic heart disease) anatomical defect [Greenspan 1991]. The source of these organisms has historically been attributed to the tonsils, periapical infections of the teeth, or infection of the gums which shed bacteria into the circulation following manipulation of these areas during dental care. Evidence that these organisms also colonize dental unit waterlines and are delivered in the operatory water in great numbers suggests that exogenous sources of bacteria may also play a role [Mayo *et al* 1990, Williams *et al* 1993]. Reports of endocarditis caused by nonstreptococcal dental water organisms generally considered saprophytes [Zinman *et al* 1991] suggest that these organisms may present a threat to patients at risk for endocarditis as well.

While delivery of contaminated water to a patient's mouth with the potential for ingestion or inoculation into the oral mucosa may initially seem the most serious hazard, an unrecognized and unexplored risk is represented by the aerosols. Dental procedures, especially those involving

high-speed handpieces, produce aerosols [Abel *et al* 1971, Belting *et al* 1964, Earnest *et al* 1991, Hausler *et al* 1964, Kazantzis *et al* 1961, Larato *et al* 1966, Madden *et al* 1963, Stevens *et al* 1963]. Stable dental aerosols are composed of a colloid of droplets which may be suspended many hours and carried to every corner of the dental office [Abel *et al* 1971, Belting *et al* 1964, Hausler *et al* 1964, Kazantzis *et al* 1961, Micik *et al* 1969]. Pathogenic organisms such as *Legionella* and *Mycobacterium* within aerosolized droplets may avoid dessication and remain infective for several hours, allowing the inhalation of aerosols to serve as a means of transmission [Belting *et al* 1964, Hambleton *et al* 1983, Macfarlane *et al* 1983, Muder *et al* 1986, Zuravleff *et al* 1983].

Aerosols are aspirated into the air-intake port of the dental air line apparatus. Once aerosolized organisms are within the air lines, they colonize them [Costerton *et al* 1987]. The air line biofilm has the potential to amplify the contaminants, and shed them into the lumen where they are blown into the dental office air and the patient's mouth. Very little research has been done to investigate the medical significance of air line organisms in the dental office, though they have recently been implicated in two infectious intrathoracic complications of routine dental

care [Ely *et al* 1993]. While the authors attributed these infections to bacteria aerosolized by the dental air line, others have suggested the waterline as an alternative source [Ely *et al* 1995, Mackenzie *et al* 1995].

Vacuum lines receive microbial contamination directly from the patients' mouth. These organisms also generate biofilms. Backflow of microorganisms into the disposable saliva ejector and ultimately the next patient's mouth has been reported [Watson *et al* 1993]. When the airflow through the lines suddenly decreases (as occurs when a patient seals his lips around the saliva ejector), the likelihood of this "suck-back" phenomenon occurring increases.

CONTAMINATION CONTROL MEASURES

In addressing person-to-person contagion and instrument-borne cross-infection, the dental profession and CDC recommend diligent attention to sterilization of dental handpieces, disinfection of operatory surfaces, and the use of gloves, goggles, and masks by dental professionals [Centers for Disease Control and Prevention 1993]. As these measures are adopted transmission of pathogens between patients and dental professionals will be reduced, though not eliminated [Crawford *et al* 1985, Merchant *et al* 1992].

Unfortunately, current practices do not always reflect current CDC recommendations. In 1989, fewer than 50% of dentists responding to a survey sterilized their instruments on a daily basis, and only 12.5% sterilized them between patients [Dental Products Report 1993]. In a 1991 report, 80% of dentists claimed to surface-disinfect their handpieces rather than autoclave/gas-sterilize them, and only rarely was *any* form of disinfection or sterilization applied to air-water syringes [Christensen *et al* 1991]. The latter is still true today.

CDC recommends flushing of dental waterlines as a means to control waterline microbial contamination [Centers for Disease Control and Prevention 1993]. However, this measure has minimal effect on the quality of the water delivered to the patient. Flushing may sometimes increase the intensity of bacterial contamination, with day-to-day variation in contamination levels exhibiting as great a magnitude as preflush-to-postflush variation [Gross *et al* 1976, Santiago *et al* 1994]. There are physical reasons for these findings.

The physics of laminar flow of fluids through cylindrical structures results in maximum velocity of water occurring at the lumenal center of the waterline, with the laminae juxtaposed to the biofilm-coated wall remaining stagnant

[Cutnell 1989]. Flushing merely rinses out planktonic organisms in the lumen, leaving the mature biofilm essentially undisturbed. Multiplication of organisms in the biofilm releases new flora into the lumen. The large surface area-to-volume ratio of the lines leads to generation of high concentrations of bacteria in a relatively short time.

Ultraviolet irradiation of the incoming operatory water supply is of limited value. A recent study of the effects of ultraviolet irradiation on the growth of *Legionella* and other heterotrophic bacteria in water in a circulating cooling system failed to show a reduction in bacterial numbers. This failure was attributed by the authors to the protective effects of the biofilm in which the organisms reside [Kusnetsov *et al* 1994].

Approaches to the problem of waterline contamination that are more effective than flushing or irradiation include the addition of chemical disinfectants, the use of bottled water reservoirs, bacteriological membrane filters, autoclavable operatory components (i.e. waterlines in addition to instruments), and various combinations of these. Each has potential to reduce significantly the levels of bacterial contamination in the dental water system, but all require

diligent, conscientious maintenance in order to be effective [Williams *et al* 1996]. Presently, however, few dental offices employ any of these methods. The medical community has taken the approach of installing in-line bacteriological filters between the patient and the infusion solution reservoir, with all infusion lines and reservoirs employing a single-use, disposable design. Such an approach may well become standard dental practice, as well.

SIGNIFICANCE OF HETEROTROPHIC BACTERIA

Heterotrophic bacteria have frequently been considered primarily saprophytic, with little innate pathogenic potential [Matsen *et al* 1975]. The only medical significance attributed to them was in the case of immunodeficient or otherwise compromised patients. They have always been important in water quality assessment because overgrowth of water heterotrophs will obscure the presence of "marker" pathogens (coliforms) [Enviromental Protection Agency 1987, Environmental Protection Agency 1989, Matsen *et al* 1975, Payment *et al* 1994].

An extensive study of these pigmented water bacteria in the context of hospital infections was undertaken by Matsen

[Matsen *et al* 1975]. From it, they concluded that "pigmented water-associated bacteria, with the exception of *Pseudomonas aeruginosa*, are infrequent hospital isolates of low-grade pathogenicity."

In 1987, Martin diagnosed *Pseudomonas aeruginosa* infections in two chemotherapy patients [Martin *et al* 1987]. Upon obtaining a thorough history, he discovered that they had both recently received care at the same dental clinic. Cultures of water samples obtained from the operatories in the clinic grew out organisms which were identical to those found in the lesions. Through serial sampling of the oral flora of 78 subsequent patients visiting the clinic, he found colonization by the same strain of organism for up to 10 weeks following exposure. In all the prospective cases, this colonization was asymptomatic. Recent surveys of dental clinics have revealed that dental unit water contamination by pseudomonads of a variety of species is a ubiquitous problem [Mayo *et al* 1990, Williams *et al* 1993].

In recent years there has been a growing conviction that not only *P. aeruginosa*, but virtually *all* heterotrophic bacteria are opportunistic pathogens whose delivery in potable water should be controlled. In a study by Payment, 57% of tap water samples grew out cytolytic colonies, and 6% contained

bacteria having three or more of the virulence factors assessed [Payment *et al* 1994]. Similar findings were reported by Lye, who found that 1% of all bacteria isolated from drinking water samples produced cytotoxic factors [Lye *et al* 1991]. While most of these organisms were of genera that are not traditionally associated with significant public health problems, on occasion they have been implicated in clinically significant infections. The presence of multiple virulence factors increases their disease-causing potential [Payment *et al* 1994].

During a prospective epidemiological study of gastrointestinal diseases associated with drinking water, a statistically significant univariate correlation was found between rate and duration of reported symptoms and counts of heterotrophic organisms grown from the water source [Payment *et al* 1991a, Payment *et al* 1991b]. This correlation observed in the absence of known enteropathogenic organisms is strong evidence of the clinically and statistically significant pathogenic potential of heterotrophic water microbes in healthy, immunocompetent individuals. Such hazards are accentuated for the immunocompromised patient, and the numbers of immunocompromised individuals in the general population are increasing with the rise in organ transplant recipients and AIDS patients. The counts of

heterotrophic bacteria observed in dental water are much higher than those found in unpolluted lakes and streams [LeChevallier *et al* 1987, Reinheimer *et al* 1991, Santiago *et al* 1994], being instead in the range found in dilute sewage [Gainey *et al* 1950, Reinheimer *et al* 1991]. They are well in excess of the number seen in Payment *et al*'s studies.

The literature also contains a report of endocarditis caused by nonstreptococcal dental water organisms generally considered saprophytes [Zinman *et al* 1991]. This indicates cardiovascular as well as gastrointestinal pathologies may be caused by these organisms.

SIGNIFICANCE OF *LEGIONELLA*

Legionnaire's disease, first described in 1976 following an outbreak of fulminant pneumonia in 221 individuals attending an American Legion convention, is caused by bacteria of the genus *Legionellaceae*. Infection by these organisms is responsible for an acute fibrinopurulent pneumonia histologically characterized by a hypoxia-inducing exudate of neutrophils, macrophages, and fibrin in the alveoli secondary to capillary damage. This presents clinically with a variable severity, ranging from mild pneumonia to

adult respiratory distress syndrome (ARDS) with major systemic manifestations. Following an incubation period of 2 to 10 days, high fever, nonproductive cough, chills, cephalgia, and myalgia abruptly appear. Hyponatremia, diarrhea and neurological deficits are also present in 50, 36, and 25 percent of patients, respectively [Fang *et al* 1989, Reingold 1988, Winn *et al* 1981]. This may progress to empyema, pneumothorax, and respiratory failure with an untreated mortality of 10 to 20 percent. In 1985 alone there were 75,000 cases of clinical legionellosis in the U.S., with over 11,000 resulting in death [Schleicher *et al* 1995]. *Legionella* pneumonia may affect both humans and nonhuman mammals [Fabbi *et al* 1993].

The route of infection has long been believed to be via inhalation of aerosols generated from contaminated water sources such as air conditioners, showers, decorative fountains, humidifiers, supermarket produce sprayers, etc. [Bolin *et al* 1985, Dondero *et al* 1980, LaMaine *et al* 1990, McDade *et al* 1977, Stout *et al* 1993, Winn *et al* 1988]; however, more recently it has been shown that ingestion, aspiration, and direct inoculation (including via irrigation of surgical wounds) play significant roles, as well [Blatt *et al* 1993, Lowry *et al* 1991, Lowry *et al* 1993].

Following invasion, *Legionella* are phagocytized by neutrophils and macrophages [Nash *et al* 1988]. Once inside they multiply within the cells. While antibodies enhance phagocytosis, they do not decrease the intracellular survival of the organisms. Activated macrophages do kill the organism suggesting a role for cell-mediated immunity in recovery from and prevention of the disease [Nash *et al* 1988]. Intracellular multiplication of *Legionella* results in cell death and lysis followed by release of host and bacterial enzymes. At least two cytotoxins, an endotoxin-like substance, and numerous extracellular enzymes which have been described, but not yet fully characterized, are believed to be responsible for the lung tissue damage [Fang *et al* 1989, Reingold 1988, Winn *et al* 1981]. In addition to Legionnaire's disease and Pontiac Fever, *Legionella* has been implicated in endocarditis, myocarditis, pericarditis, pyelonephritis, pancreatitis, sinusitis, post-pericardiotomy-like syndrome, and abscess formation [Bernstein *et al* 1991, Camara-Gonzalez *et al* 1993, Kaye *et al* 1991, Tompkins *et al* 1988].

Legionellaceae have been shown to invade and multiply intracellularly within a number of free-living protozoa, including *Acanthamoeba, Tetrahymena, Hartmannella,* and

Naegleria, with the amoebae being the most significant natural hosts [Anand *et al* 1983, Breiman *et al* 1990, Fields *et al* 1984, Fields *et al* 1990, Harf *et al* 1987, Henke *et al* 1986, Newsome *et al* 1985, Rowbotham *et al* 1986, Tyndall *et al* 1982, Wadowsky *et al* 1988]. *Legionella* survive and multiply within these protozoa; in fact, their growth in the environment in the absence of protozoan hosts has not been documented, suggesting that such intracellular sequestration is a prerequisite for survival of the genus [Fields *et al* 1989, Fields *et al* 1993, Wadowsky *et al* 1988]. *Legionella's* role as an intracellular symbiote/pathogen of both protozoan and mammalian hosts is representative of an ecological interaction only recently being appreciated and investigated by the scientific community. Barry Fields postulated "some intracellular pathogens of higher vertebrates may have acquired the ability to infect these organisms by first adapting to intracellular life in protozoa. Predation by free-living protozoa appears to be an ideal selective pressure for the evolution of these intracellular bacteria. It is tempting to imagine that they subsequently acquired mechanisms for infecting higher eukaryotic cells." He concedes, however, that this "contradicts a central doctrine of evolution, that more highly evolved organisms tend to become more specialized...studies of the evolution of

Legionellae may have implications concerning the origins of intracellular bacteria" [Fields *et al* 1993].

The epidemiological efforts to track down the sources of *Legionella* infections have employed a variety of different methods. While culture on specialized media remains the gold standard for detection of the organisms, other more rapid techniques are more often employed. Direct fluorescent antibody (DFA) screen of both clinical and environmental specimens is widely used [Broome *et al* 1979]. The literature reports on the sensitivity and specificity of this technique are mixed. Reports of 30-80% sensitivity and 30-95% specificity suggest that reliability of results may be largely dependent on the skill of the individual technician [Tompkins *et al* 1993]. More recently, a variety of molecular biology techniques have been employed.

Nucleic acid hybridization based on cDNA probes complementary to specific regions of 16S RNA sequences has been used, with reported sensitivity of 56-74% and 100% specificity [Edelstein *et al* 1987, Tompkins *et al* 1993]. A head-to-head comparison with DFA resulted in 7 of 11 positive clinical specimens being detected by probe, while only 4 of 11 were DFA positive [Pfaller *et al* 1988, Tompkins

et al 1993]. These probes have not been used to evaluate contamination of water samples.

Polymerase chain reaction (PCR) has also been employed, using primers based on *Legionella* specific 5S and 16S RNA and *L. pneumophila* specific *mip* and *pro* sequences. Sensitivity and specificity of 100% are attainable using this technique, including detection of viable-nonculturable specimens [Atlas *et al* 1995, Loutit *et al* 1990, Miller *et al* 1993, Tompkins *et al* 1993, Yamamoto *et al* 1993]. The procedure has been applied to both enviromental and clinical specimens [Tompkins et al 1993]. A similar technique, based on the ligase-chain reaction (LCR) is also being developed [Tompkins et al 1993].

Once a *Legionella*-positive source of contamination is identified, confirmation of the presence of the strain responsible for the infection is imperative. There are over 30 *Legionella* species and more than 40 distinct *L. pneumophila* subtypes described in the literature. Given the ubiquitous nature of these organisms in water and moist environments, it is common to isolate several different *Legionella* species/strains from a single plate of primary culture [Barbaree *et al* 1993]. Subtyping procedures are necessary to make the link between the environmental and

clinical isolates. Methods used include polyclonal and monoclonal serological techniques [Barbaree *et al* 1993, Cherry *et al* 1978, Joly *et al* 1986], plasmid analyses [Aye *et al* 1981, Brown *et al* 1985], electrophoretic alloenzyme typing [Barbaree *et al* 1993], ribotyping [Grimont *et al* 1989, Mao *et al* 1989], restriction length polymorphism (RFLP) analysis [Pang *et al* 1990, Saunders *et al* 1990], nucleotide probes of DNA digests [Stout *et al* 1988], pulsed-field gel electrophoresis of DNA digests [Barbaree *et al* 1993, Ott *et al* 1991], 16SrRNA-based speciation [Hookey *et al* 1995], and repetitive element PCR (repPCR)[Georghiou *et al* 1994, vanBelkum *et al* 1993].

High levels of exposure to *Legionella* among dental professionals are well documented. Serological studies from Europe and the United States show seropositive rates among dental clinic staff are substantially higher than that of the general population [Fotos *et al* 1985, Luck *et al* 1993, Reinthaler *et al* 1988]. Differences among the staff have been related to their level of exposure to aerosols generated in the operatory [Reinthaler *et al* 1988]. While most of these exposures apparently result in asympomatic seroconversions or undiagnosed Pontiac Fever manifestations (largely indistinguishable from other "flu-like" symptoms), a fatal case of Legionnaire's disease in a dentist was

linked to his dental operatory water [Mackenzie *et al* 1994]. Surveys of the dental operatory water demonstrate that *Legionella* are present at high intensities [Luck *et al* 1993]. Given the high intensity of *Legionella* contamination of dental water, and the fact that no source of infection is established in the vast majority of all cases of Legionnaire's disease [Stout *et al* 1992], it seems reasonable that exposure in the dental operatory is the source of some of these cases. However, prior to the studies reported in this dissertation, a direct comparison of *Legionella* prevalence and intensity in dental water as opposed to other potable water sources had not been made. Thus, while levels of *Legionella* contamination were known to be high, no clear conclusions regarding their significance as a possible source of exposure could be reached.

SIGNIFICANCE OF ENDOTOXIN

Since dental unit water commonly contains high concentrations of gram negative organisms, it is correspondingly likely to be a rich source of endotoxin.

Endotoxin, a heat-stable toxin associated with gram negative bacterial cells, is composed of lipopolysaccharides (LPS).

These amphilic molecules consist of three regions, including O-specific polysaccharide (the somatic O antigen), core polysaccharide, and lipid A. The lipid A region is associated with the toxic effects induced and the other two regions confer the serological specificity, which is widely variable.

The mammalian gastrointestinal tract is heavily colonized by a variety of gram-negative organisms existing symbiotically with their host as normal gut microflora. They produce endotoxin which is absorbed across the mucosa into portal venous blood following Michaelis-Menton kinetics reaching levels of 1 EU/mL [Jacob *et al* 1977, Nolan *et al* 1977, Tokyay *et al* 1993]. The endotoxin then travels to the liver where it is removed from circulation via a biphasic clearance consisting of uptake by the reticuloendothelial system (RES) and modification of the endotoxin to a lower density form [Mathison *et al* 1979, Premaratne *et al* 1995]. This continuous uptake of endotoxin appears to serve a vital physiological function in priming and maintaining optimal phagocytic activity in the RES [Van Leeuwen *et al* 1994]. Hence, endotoxin is a normal constituent of portal blood; however, it is only found systemically in disease states [Jacob *et al* 1977].

Pathological endotoxin exposure may be seen in conditions with decreased RES function such as liver disease, or conditions with increased absorption that overwhelms the RES such as burns, trauma, radiation, gastrointestinal disease [Van Leewen *et al* 1994]. However, endotoxemia is most significantly associated with gram-negative infection and sepsis. The presence of these organisms in an abscess or as a bacteremia results in circulation of endotoxin in the systemic blood. Other modes of exposure such as inhalation [Castellan *et al* 1987, Teeuw *et al* 1994] have only recently been appreciated. Little is known about the significance of aspiration, ingestion, mucosal or dermal exposure. Gram negative organisms in the biofilms of plumbing and climate-control ductwork have the potential to contribute medically significant quantities of endotoxin to their surroundings [Costerton *et al* 1987, Hugenholtz *et al* 1992, Teeuw *et al* 1994].

Many of the pathogenic effects of gram negative bacteria are mediated by the endotoxin [Berczi *et al* 1993, Natanson *et al* 1994] to the extent that the clinical syndrome present during gram negative sepsis may occur in the absence of bacteremia, and is referred to as endotoxemia or endotoxic shock [Danner *et al* 1991, Graham *et al* 1994]. This

dependence on the toxin rather than the organism explains why septic shock may occur even following successful antibiotic treatment of the active infection. This distinction may be hard to insist upon, given the cell-wall associated nature of the endotoxin. Nevertheless, endotoxin may be present even in the absence of viable, intact bacteria. A 3- to 20-fold increase in endotoxin concentration is observed following antibiotic treatment both *in vitro* and *in vivo* [Hurley 1992, Shenep *et al* 1988]. This is seen with both beta-lactam and nonbeta-lactam antibiotics, and occurs after an unexplained delay following antibiotic exposure. The mechanism of this increase is unknown, but both lytic and non-lytic mechanisms were suggested by the authors.

Much of the pathology seen in endotoxemia is primarily due to the host response to the molecule rather than direct injury by the endotoxin itself (though endotoxin has been shown to directly damage pulmonary endothelium [Martin *et al* 1992]). Endotoxin stumulates the production of interleukin 1, 6 and 8 (IL1, IL6, IL8) [Levi *et al* 1993, vanDeventer *et al* 1990], tumor necrosis factor alpha (TNF) [Beutler *et al* 1987], and the activation of the alternative complement pathway and extrinsic coagulation cascade [Graham *et al* 1994, Shoemaker *et al* 1987]. Interleukin 1 directly causes a

pyrogenic reaction and muscle proteolysis [Graham *et al* 1994, Suffredin *et al* 1989]. The cytokines, especially TNF, damage the endothelium of the lung parenchyma and vasculature, yielding an increase in vascular permeability [Graham *et al* 1994, Herbert *et al* 1992, Horgan *et al* 1993, Mannel *et al* 1987]. The resulting pulmonary edema may progress to adult respiratory distress syndrome (ARDS) followed by multisystem organ failure (MSOF), which is lethal in 60-90% of cases [Graham *et al* 1994, Herbert *et al* 1992, Horgan *et al* 1993, Martin *et al* 1992]. Experimental injection of endotoxin into healthy volunteers elicits all the symptoms of endotoxemia [Herbert *et al* 1992, Suffredin *et al* 1989, vanDeventer *et al* 1990]. Interestingly, while TNF appears to be the molecule principally responsible for the pathogenesis of endotoxin-induced tissue injury, administering TNF prior to administration of a lethal dose of endotoxin to laboratory animals actually protected them from death or injury [Alexander *et al* 1991]. The reason for this apparent paradox is unclear.

Inhaled endotoxin significantly lowers spirometric values in otherwise healthy subjects [Castellan *et al* 1987]. The likely mechanism is the same as that described for endotoxemia. Obviously, breathing endotoxin-tainted air has serious implication for exacerbation of chronic obstructive

pulmonary disease and asthma in individuals with pre-existing conditions. The clinical significance in healthy individuals is not known. However, recent studies linking airborne endotoxin to "sick building syndrome" [Teeuw *et al* 1994] would suggest that even uncompromised individuals are at risk for the development of medical conditions due to inhaled endotoxin.

In the study correlating airborne endotoxin to "sick building syndrome", 19 mechanically ventilated buildings were divided into "sick" and "healthy" groups, based on symptom prevalence (>15% or <15%, respectively). Airborne endotoxin levels were 6 to 7 times higher in sick buildings than in healthy ones (254 vs 46 ng/m^2), both of which were higher than naturally ventilated buildings (35 ng/m^2). These findings suggested a significant contribution of airborne endotoxin to the etiology of sick building syndrome [Teeuw *et al* 1994]. Biofilm has been found in air conditioning systems, and may represent the source of this endotoxin [Hugenholtz *et al* 1992]. The finding is important to the work reported here: dental waterlines have lush biofilms within them [Mayo *et al* 1990], and tremendous aerosols are generated during the course of routine dental procedures [Abel *et al* 1971, Belting *et al* 1964, Earnest *et al* 1991, Hausler *et al* 1964, Kazantzis *et al* 1961, Larato *et al* 1966,

Madden *et al* 1993, Stevens *et al* 1963]. If dental water contains endotoxin, these aerosols could be contributing to the development or exacerbation of numerous medical conditions, not the least of which is periodontitis [Trope *et al* 1995, Yoshinuma *et al* 1994].

SUMMARY

Infectious complications of dental care may involve nearly any site on the patient, both anatomically and temporally distant from the dental care. A majority of these conditions may be attributed to the dental water as the source of exposure, either through ingestion, inoculation into the operative site, or inhalation of the aerosols generated by dental procedures. The full extent of the public health and occupational health implications of these complications is not yet appreciated.

Within this dissertation is presented a characterization of what may be three of the most prevalent, and significant, microbial contaminants encountered in a trip to the dentist: heterotrophic bacteria, *Legionella,* and endotoxin.

Chapter 1

Microbial contamination of dental unit waterlines: prevalence, intensity, and microbiological characteristics

INTRODUCTION

Microbiological contamination of dental water systems has been recognized for nearly a third of a century [Abel *et al* 1971, Blake 1963, Clark *et al* 1974, Fitzgibbon *et al* 1984, Martin *et al* 1987, Micik *et al* 1969, Miller *et al* 1991]. Initially, studies focused on oral microbiota and the significance of their aerosolization [Holbrook *et al* 1978, Micik *et al* 1969, Miller *et al* 1971]. Later, at a time when dental water microbes were widely regarded as harmless nonpathogenic saprophytes, the emphasis shifted to concern over the aesthetic aspects of delivering large numbers of bacteria of any sort to patients [Abel *et al* 1971, Clark *et al* 1974, Fitzgibbon *et al* 1984, McEntegart *et al* 1973]. Contemporary thought is that these saprophytic organisms *do* have pathogenic potential and are, in fact, significant in human diseases, and modern dental microbiology reports

reflect this concept [Pankhurst *et al* 1993, Mayo *et al* 1993, Williams *et al* 1993].

Since the first recognition that dental water microbes may represent a health concern, numerous changes in hygienic practices and instrument design have occurred. This chapter reports the profiling of bacterial contamination of dental unit water from operatories in the western United States to evaluate what effect, if any, these changes in the practice of dentistry might have had on microbiological profiles of the water in dental lines. The scope of the contamination and the characteristics of the microbiological populations involved suggest that there is much room for improvement.

MATERIALS AND METHODS

In 1992, dental unit waterline (DUWL) samples were collected from 116 three-way syringe lines, 54 high-speed handpieces and 12 scaler lines, from 150 operatories at 54 sites in Washington, Oregon and California. The sites of sampling were selected with a conscious effort to obtain representative samples from a variety of dental practice types, including private practices, university-based clinics, new practices, and well established practices.

Collections were made by collaborating dentists and by colleagues at the University of Washington. Samples were taken at various times during the working day, but sampling was avoided in the early morning when stasis of water after the weekend or overnight can lead to artificially high concentrations of bacteria. No attempt was made to flush lines immediately before sampling as the aim was to collect water representative of that issuing from instruments during typical procedures.

However, in some instances specified here, tests were conducted before and after a defined flushing period. Office faucet water samples were also collected from 11 sites for comparison with the corresponding DUWL. Seven additional operatories were included whose water was supplied by distilled water reservoirs.

Water samples were handled and processed according to standard practices for water quality evaluation using heterotrophic bacterial plate counts [American Public Health Association 1985]. Samples (2 to 3 mL) were collected aseptically in sterile 12 X 75 polystyrene tubes, avoiding any contact between the instrument parts and the collection tube during the collection. Samples were shipped overnight to the laboratory, usually but not always, with a cold pack.

Cultures were generally established within 36 hours. Some late-arriving samples were refrigerated overnight before culture. Delays longer than 36 hours are known to lead to artifactual declines or increases in bacterial counts depending on the flora, although these changes are severely limited by refrigeration.

Log dilutions were made in sterile water for culture on TSA plates and incubated at room temperature (~20C) for 96 hours before counting. Scores below 25 colony-forming units (cfu) on undiluted samples were recorded for statistical reasons as too few to count (TFC). Microbiological typing of colonies selected from the bacterial growth was conducted according to the procedures described by the American Society of Microbiology, Clinical Microbiology Manual [American Society for Microbiology 1989]. These involve the classical microbiological techniques of recording microscopic and colony characteristics, gram stain reactivity, growth on selective media and use of biochemical typing profile reagents. The latter involved testing for nitrate reduction, tryptophanase activity, glucose fermentation, arginine dihyrolase activity, urease activity, esculin hydrolysis, gelatinase activity, beta-galactosidase activity, and assimilation of D-glucose, L-arabinose, D-mannose, D-mannitol, N-acetyl-D-glucosamine, maltose, D-

gluconate, caprate, adipate, L-malate, citrate, and phenylacetate by way of inoculation and incubation of convenient, commercially available multi-cupule typing strips (API kits, Sherwood Medical, Plainview, New York).

RESULTS

Seventy-two percent of DUWL samples contained bacterial populations that would qualify them as "unfit for human consumption" (>500 colony-forming units per milliliter [cfu/mL]), according to U.S. Army standards [Abel *et al* 1971] (Figure 1). Mean heterotrophic colony-forming unit counts were 49,700 (σ = 156,200), with a maximum of 1,200,000 cfu/mL for the three-way syringe samples, and 72,500 (σ = 140,300), with a maximum of 550,000 cfu/mL for high-speed handpiece lines. Only 28 DUWL samples yielded plate counts classified as TFC, whereas nine of 11 office faucet sample counts were TFC. Only one faucet sample qualified as unfit for consumption (Figure 2).

Most faucet samples showed zero growth, even after prolonged incubation. Samples from 12 ultrasonic scalers showed a similar pattern of severe microbial contamination (mean 19,800 cfu/mL, σ = 37,300). No trends emerged with regard to

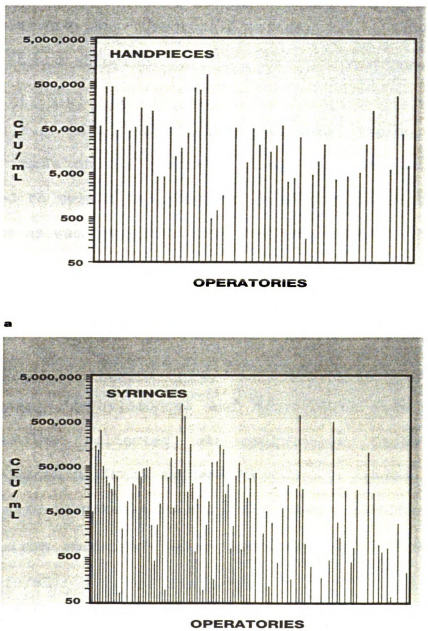


Figure 1. Histograms showing heterotrophic bacterial plate counts (vertical bars) in cfu/mL in dental unit water samples from (a) 116 individual high-speed handpieces and (b) 54 individual three-way syringes. Note the wide range of concentrations (on logarithmic scale) and the small proportion of samples with bacteria levels in the "potable" range of 500 cfu/mL or below. Reprinted by permission of ADA Publishing Co., Inc. [Williams *et al* 1993].

types or models of dental units and the degree of contamination of water samples, nor were there noticeable differences between geographic sites, or influences of collection and shipping.

Although systematic quantitative microbial typing profiles were not done on all samples, colony form characteristics were used to select and speciate as many distinct bacterial organisms as was practical from the DUWL samples (Table 1). *Pseudomonas* spp. predominated in most samples, but the most usual finding was a variety of identifiable genera on TSA cultures.

Pseudomonas, Staphylococcus and *Micrococcus* were cultured from samples collected at operatories using closed distilledwater delivery systems (Figure 3). Counts in these samples were comparable to those from units receiving water derived from municipal water supplies (mean 28,300, σ = 35,800 cfu/mL); only one sample was in the TFC category.

Flushing of handpieces continuously for two minutes lowered cfu/mL, reducing counts in most cases to a third or less of the pre-flush concentrations. However, they were not reduced to TFC in any instance.

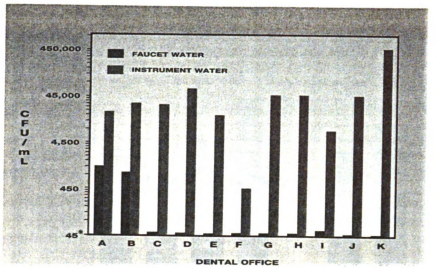


Figure 2. Histogram showing heterotrophic bacterial plate counts in paired faucet and dental unit water samples. Log scale shows the wide range of bacterial concentrations in the dental water with only one in the "potable" range of 500 cfu/mL or below. All except one of the faucet samples had levels in the "potable" range. Reprinted by permission of ADA Publishing Co., Inc. [Williams *et al* 1993].

Table 1. Organisms identified in dental water samples

Bacteria:	
Pseudomonas aeruginosa	*Flavobacterium indologenes*
Pseudomonas cepacia	*Staphylococcus saprophyticus*
Pseudomonas fluorescens	*Staphylococcus capitus*
Pseudomonas vesicularis	*Staphylococcus warneri*
Pseudomonas paucimobilis	*Staphylococcus* spp.
Pseudomonas pickettii	*Legionella pneumophila*
Pseudomonas acidovorans	*Legionella* spp.
Pseudomonas testosteroni	*Ochromobacterum anthropi*
Pseudomonas stutzeri	*Alcaligenes denitrificans*
Xanthomonas maltophilia	*Acinetobacter* spp.
Pasteurella haemolytica	CDC Group IVc-2
Pasteurella spp.	
Achromobacter xyloxidans	**Fungi:**
Micrococcus luteus	*Alternaria* spp.
Klebsiella pneumoniae	*Penicillium* spp.
Bacillus spp.	*Scopulariosis* spp.
Streptococcus spp.	*Cladosporium* spp.

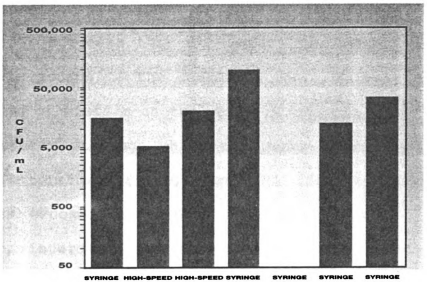


Figure 3. Histogram showing heterotrophic bacterial plate counts on samples from instruments receiving water from distilled water supplies. Log scale depicts wide range of bacterial concentrations in the dental unit water. Only one sample's bacterial level falls within "potable" range. Reprinted by permission of ADA Publishing Co., Inc. [Williams *et al* 1993].

DISCUSSION

These findings are consistent with previous reports and show that there has been no demonstrable improvement in water quality in the dental operatory in spite of significant changes in the practice of dentistry and infection control in recent years [Clark *et al* 1974, Fitzgibbon *et al* 1984, Martin *et al* 1987].

The difference between the microbial quality of water from office faucets and operatory samples (at least 3 orders of magnitude) suggests that the waterlines themselves are the proximate source of the contamination. This is due to the formation of biofilm on the walls of the fine bore tubing of the operatory through which the water flows. As reviewed above (Literature Review, page 10), these biofilms form on surfaces exposed to aqueous flow and consist of complex, dynamic, interdependent microbial populations of bacteria, protozoa, fungi, and even occasionally nematodes [Costerton *et al* 1987]. Adherent organisms have their origins in both the incoming municipal water supplies as well as in material sucked back into the lines from the patients. Biofilms trap nutrients, offer varied oxygenation microenvironments, and limit influx of biocides, making them ideal sites for the persistence of resident microbes and the production and release of planktonic forms to pioneer adherence at surfaces downstream. Some of the variation in levels of contamination between water samples seen in this study may be attributed to "clumps" of organisms being dislodged during manipulation of the DUWL in some samples and not others. The majority of heterotrophic water bacteria do not grow on tryptic soy agar, requiring specialized media or even being entirely unculturable *in vitro* [American Society for Microbiology 1989]. As a result, the levels of

microbial contamination presented in this chapter represent underestimations of the extent of contamination actually present.

Prior to this study few attempts at speciation had been reported [Clark *et al* 1974, Fitzgibbon *et al* 1984, Martin *et al* 1987]. Nowhere in the literature is there reported so extensive a characterization of dental unit water flora as is presented in this chapter. The majority of organisms identified were gram negative pseudomonads. These "heterotrophic bacteria" or "pigmented water associated bacteria" were historically considered to be saprophytic and of little medical consequence, except to the already severely debilitated patient [Matsen *et al* 1975]. In recent years, however, there has been a growing appreciation that many of these bacteria are actually opportunistic pathogens, and their growth in potable water supplies can have significant disease consequences even in consumers with no immunological impairment [Payment *et al* 1994]. Heterotrophic organisms have been implicated in endocarditis and gastrointestinal maladies [Payment *et al* 1991a, Payment *et al* 1991b, Zinman *et al* 1991] and produce numerous virulence factors, antibiotic resistance genes, and cytotoxins [Lye *et al* 1991, Payment *et al* 1994]. In addition to the medical significance of the high levels of

microbial fouling demonstrated in this study, the putrid odor and bad taste and texture associated with dental operatory water is a consequence of the types and quantities of organisms seen here.

Some organisms identified are widely recognized as pathogens. *Pseudomonas* and *Klebsiella* have dental significance [American Public Health Association 1985, American Society for Microbiology 1989, Costerton *et al* 1987, Martin *et al* 1987, Miller *et al* 1991, Slots *et al* 1988], and *Staphlylococcus, Acinetobacter, Nocardia, Pasteurella, Serratia, Pseudomonas, Klebsiella, Legionella,* and others are associated with important medical conditions as a result of infection and production of allergens and toxins, including endotoxin [American Society for Microbiology 1989, Clark *et al* 1974, Martin *et al* 1987, Miller *et al* 1991]. *Legionella*, one of the more significant in terms of dental water contamination, will be discussed in greater detail in subsequent chapters.

Dispensing either primary or opportunistic pathogens into dental patients' mouths may be responsible for post-dental care complications, including many which have no oral component. Administration of water with these characteristics is clearly undesirable, and has legal

consequences [Zinman 1991]. In addition to exposing dental patients to water-borne pathogens, dental professionals also receive occupational exposure [Clark *et al* 1974, Fotos *et al* 1985, Mackenzie *et al* 1994, Reinthaler *et al* 1987].

None of the solutions to the problem of dental water contamination discussed in the literature review have come into general use [Centers for Disease Control and Prevention 1993, Clark *et al* 1974, Kusnetsov *et al* 1994, Martin *et al* 1987, Miller *et al* 1991, Santiago *et al* 1994, Williams *et al* 1996]. One measure growing in popularity is to use commercially available bottled water or saline reservoirs. Our finding of contamination on the order of that seen with municipally supplied operatories suggests that even waterlines attached to such reservoirs become rapidly colonized by a biofilm of patient-derived organisms or organisms due to a failure to maintain the system aseptically. This negates the initially perceived benefit of the sterile water supply. Contamination of the lines often extends to the reservoir, probably as a result of poor maintenance and improper handling and filling procedures. In the absence of residual antimicrobials in the water, the extent of contamination may become massive in a short time [Williams *et al* 1993, Williams *et al* 1994].

CONCLUSION

Microbial contamination of dental water continues to be widespread and extensive. Organisms identified included both opportunistic and primary pathogens. The presence in dental waterlines of high numbers of these organisms, some of which are patient-derived, needs to be addressed in the context of current concerns over handpiece sterilization and infection control practices in the dental office. Until the issue is addressed satisfactorily, infection control practices based on more widespread and frequent sterilization of handpieces will remain flawed. The value of this practice in reducing risks of patient-to-patient transmission of pathogens is unquestionable. Our results suggest, however, that sterile instruments may become heavily contaminated with bacteria as soon as the handpieces are attached to the DUWL.

Chapter 2

Legionella Contamination of Dental-Unit Water

INTRODUCTION

The literature contains reports that dentists, dental students, and dental staff have higher rates of respiratory tract infections, common colds, and other respiratory ailments than the general public [Burton *et al* 1963, Cuthbertson *et al* 1954, Carter *et al* 1953, Mandel *et al* 1993, Scheid *et al* 1982]. Both viruses and bacteria, including *Legionella,* have been proposed as possible agents involved in the pathogenesis of respiratory symptoms in dental personnel [Paszko-Kolva *et al* 1991]. Higher rates of seropositivity for *Legionella* antibodies have been found among dental personnel than among the general public [Fotos *et al* 1985, Paszko-Kolva *et al* 1993, Reinthaler *et al* 1988]. The aerosols generated in dental operatories during the course of routine dental care are a likely source of exposure to *Legionella* spp. Water-cooled, high-speed

handpieces generate stable aerosols [Abel *et al* 1971] that may contain *Legionella* spp. As discussed in the literature review and the preceeding chapter, the complex design of the dental operatory with its long runs of tubing and intermittent water flows results in the stagnation of water within the water lines, where bacteria, including *Legionella* spp., can proliferate within a biofilm [Pankhurst *et al* 1993].

Legionella pneumophila and other *Legionella* spp. have been isolated from dental water [Pankhurst *et al* 1990, Reinthaler *et al* 1986]. These organisms are often difficult to isolate because of overgrowth by other microorganisms and because *Legionella* spp. often are sequestered within amoebae [Anand *et al* 1983, Breiman *et al* 1990, Fields *et al* 1990, Fields *et al* 1993, Fields *et al* 1984, Harf *et al* 1987, Henke *et al* 1986, Newsome *et al* 1985, Rowbotham *et al* 1986, Tyndall *et al* 1982, Wadowsky *et al* 1988]. As a result, detection of *Legionella* spp. by viable-culture methods frequently gives variable results even from sources believed to be responsible for disease outbreaks [Miller *et al* 1993]. Historically, culture has been the first step in studying *any* bacteria. Only recently has there been a growing recognition of the importance of viable-nonculturable forms of many if not all environmental organisms [Atlas *et al*

1988, Byrd *et al* 1991, Chmielewski *et al* 1995, Davies *et al* 1995, England *et al* 1995, Gribbon *et al* 1995, Jacob *et al* 1993, Oliver *et al* 1995, Rahman *et al* 1996, Servis *et al* 1995,]. The viable-nonculturable form of *Legionella* may be clinically significant: to date there has never been a clinical isolate of *Legionella* obtained from a case of Pontiac Fever, suggesting that the disease may result from an allergic reaction to bacterial components rather than active infection. Because of the difficulty of culturing these organisms, direct fluorescent-antibody tests and PCR detection of *Legionella* spp. have been recommended for epidemiological investigations [Broome *et al* 1979, Miller *et al* 1993, Tomkins *et al* 1993]. These approaches have proven valuable when standard culture techniques were unsuccessful in tracing the source of *Legionella*. One such example was a 1992 outbreak of Pontiac fever in the United States which was linked to a resort hot tub by using PCR techniques [Miller *et al* 1993]. Currently, the vast majority of cases of community-acquired pulmonary legionellosis occur sporadically rather than epidemically, and the sources are never identified [Stout *et al* 1992]. The presence of *Legionella* in dental water may represent a previously unrecognized but important element of the medical history of a proportion of clinical legionellosis cases.

In this chapter, the prevalence and intensity of *L. pneumophila* and other *Legionella* spp. in dental-unit water samples was compared with that in potable water samples using the PCR-gene probe detection procedure described by Mahbubani et al. [Mahbubani *et al* 1990]. The hypothesis was that the prevalence and/or intensity of *Legionella* contamination of dental water would be higher than that of randomly collected potable water samples. Furthermore, it was considered likely that a molecular probe-based test would reveal higher rates of contamination than had been detected in previous studies that employed traditional culturing techniques.

MATERIALS AND METHODS

Samples

In 1993, 119 dental-unit water samples were collected from 28 dental facilities located in the United States (California, Massachusetts, Michigan, Minnesota, Oregon, and Washington), China, and Venezuela. Included in the study were both institutional clinics and private practices. Samples included water from high-speed drill handpiece lines, dental-syringe lines, and scaler lines. For

comparison, 76 potable water specimens were also collected through convenience sampling of domestic and institutional facilities and water fountains in Michigan and California. Water samples of 50 to 100 ml were collected in sterile containers and shipped on ice to the laboratory.

PCR detection of *Legionella* spp.

The PCR method detects all species of the genus *Legionella*, including viable nonculturable organisms that are not detected by conventional selective cultivation. It also identifies the presence of *L. pneumophila* and permits a semiquantitative evaluation of the intensity of contamination.

Each water sample was filtered through a Durapore filter to trap bacterial cells, and the trapped bacteria were lysed by treatment with EnviroAmp lysing reagent (Perkin Elmer-Roche Molecular Systems, Nutley, N.J.). An aliquot of the sample was transferred to a reaction vessel for amplification of the diagnostic gene sequences. The EnviroAmp detection kit was used for PCR amplification and gene probe detection. This kit includes PCR buffer, *Taq* DNA polymerase, and biotinylated primers for amplification of a genus-specific region of the *Legionella* 5S rRNA gene (5'-poly-dT-GCGCCAATGATAGTGTG-3' and 5'-poly-dT-GCGCCGATGATAGTGTG-3') and of

the *L. pneumophila mip* gene (5'-GCATTGGTGCCGATTTGG-3' and 5'-GCTTTGCCATCAAATCTTTCTGAA-3'/5'-GTTTTGCCATCAAATCTTTTTGAA-3'). A Quarterbath (Inotech Biosystems, Inc., Lansing, Mich.) thermal cycler was used for amplification with a 30-cycle program of 0.5 min at 95C for denaturation and 1 min at 63C for primer annealing and DNA extension.

The PCR-amplified 5S rRNA and *mip* DNA sequences were detected by reverse dot blot strip analysis with an immobilized probe. Specific probes complementary to internal sequences of the amplified regions are immobilized on nylon membrane strips in the detection kit. Biotinylated PCR products generated by amplification with biotinylated primers are specifically hybridized to the immobilized probes. After stringent washing of the strips, the presence of hybridized biotinylated PCR products are detected by incubating the strips with a streptavidin-horseradish peroxidase conjugate, washing, and adding the substrate for horseradish peroxidase. A blue dot appearing on the nylon membrane indicates the presence of bound PCR product.

An internal positive control (IPC) is included as a means for detecting poor amplification or hybridization. The IPC is a synthetic DNA sequence that is coamplified by the primers for the *mip* gene, and its template is included in

the EnviroAmp PCR mixture. When the IPC fails to yield the positive blue dot, it indicates poor amplification such as may be caused by the presence of inhibitors of PCR in the environmental specimen. In an effort to overcome this inhibition, aliquots of the samples were diluted 1:10 and processed in parallel with the undiluted aliquots. The positive control also provides a semiquantitative basis for estimating the number of *Legionella* cells in the sample. The IPC corresponds to 1,000 copies of the *mip* gene sequence prior to amplification, and thus the intensity of the sample hybridization signal can be graded on a scale to determine the relative number of *Legionella* cells.

An internal negative control is also included in the kit as a means of ensuring appropriate hybridization stringency. The positive control probe is perfectly complementary to a sequence in the IPC amplification product. The negative control probe has a 1-bp mismatch with this sequence. When the hybridization reactions are done correctly, the PCR product generated from amplification of the IPC will hybridize with the positive control probe but not with the negative control probe. The hybridization conditions have been optimized in this system to be stringent enough to allow detection of a 1-bp mismatch between the probe and a PCR product.

An agarose gel demonstrating the PCR products generated using this system is shown in Figure 1. The 108, 168, and 135 base pair products represent the amplified 5S rRNA, *mip*, and IPC sequences, respectively. The use of stringent

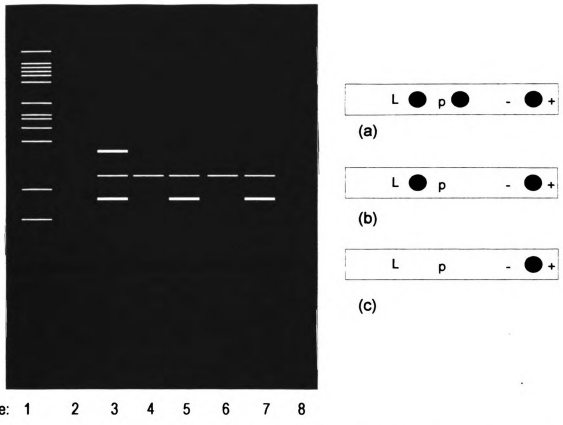


Figure 1. Schematic diagrams of an agarose gel (left) and reverse dot-blots (right) of EnviroAmp™ PCR products. Lane 1 is the molecular marker, 2 and 8 are blank, 3 is positive for *L. pneumophila* (the corresponding dot-blot is (a)), 4 and 6 are negative for *Legionella* (the corresponding dot-blot is (c)), and 5 and 7 are positive for non-*pneumophila Legionella* (the corresponding dot-blot is (b)). The 108, 135, and 168 base pair bands represent the amplified 5S rRNA, IPC, and *mip* sequences, respectively.

hybridization during the detection of these sequences allows nonspecifically amplified products to be disregarded as they are not visualized on the reverse dot blot.

RESULTS

Legionella spp. were detected by the PCR amplification-DNA gene probe method in 89% of the dental-unit water samples(Figure 2), and *L. pneumophila* was detected in 11%. Of the 119 dental-unit water samples, 63% contained concentrations of *Legionella* spp. of 1,000 to 10,000 organisms per ml, whereas of 76 domestic potable water samples, only 19% contained concentrations of *Legionella* spp. of 1,000 to 10,000 organisms per ml (Table 1). Fifty-three percent of domestic potable water samples had detectable levels of *Legionella* spp. (Figure 2), but <2% of domestic potable water samples tested positive for *L. pneumophila*. *L. pneumophila*, when present, was in the range of the lowest detectable concentrations. None of the domestic potable water samples contained concentrations of *Legionella* spp. of >10,000 organisms per ml; however, 12% of the dental-unit waters examined were in the category of >10,000 organisms per ml. None of the domestic potable water

and <2% of the dental-office water samples had concentrations of *L. pneumophila* of >1,000 organisms per ml.

The percentage of samples positive for *Legionella* spp. at different dental sites was highly variable (Table 2). For example, of 10 locations around Seattle, WA, and Portland, OR, 8 provided *Legionella*-positive dental-unit water samples, with 4 of these sites showing evidence of *L. pneumophila*. The proportion of *Legionella*-positive samples

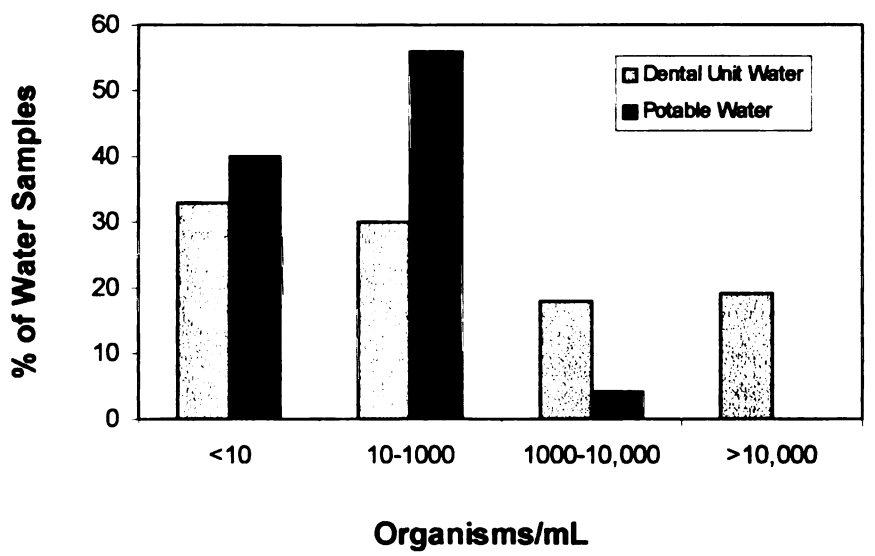


Figure 2. PCR-DNA probe test results for *Legionella* spp. in dental-unit water samples and potable water samples. This histogram shows that of the samples testing positive for *Legionella*, over 90% of potable water samples had low intensities of contamination while 37% of dental water samples had high contamination intensities of greater than 10^3 organisms/mL.

Table 1. Percent of samples positive for organisms.

	Dental Office Water		Domestic Potable water	
Organisms/mL	***Legionella* spp.**	***L. pneumophila***	***Legionella* spp.**	***L. pneumophila***
<1	11	89	47	99
1-100	26	9	33	1
100-1000	24	1	15	0
1000-10,000	28	0	5	0
>10,000	12	1	0	0

Table 2. *Legionella* spp. and *L. pneumophila* in dental water[a]

Site	# of samples tested	# positive for *Legionella*	# positive for *L. pneumophila*
1	9	2	1
2	28	19	2
3	12	0	0
4	6	4	0
5	6	6	1
6	6	0	0
7	12	12	0
8	6	6	2
9	4	4	0
10	3	2	0

[a]Samples collected from 10 locations in Portland, OR, and Seattle, WA

at each site ranged from 0 to 100%. None of the samples from two large teaching institutions was positive for *L. pneumophila*, whereas 42% from another school were positive, several at high levels (>1,000 organisms per ml).

Approximately two-thirds of all the specimens collected came from dental air-water syringe lines, with most of the remainder collected from high-speed handpiece lines. Thirty samples from scalers were processed. The samples from lines to high-speed dental handpieces were positive less frequently than those from lines to syringes, though not significantly so. Although fewer scaler line samples were evaluated, the prevalence of contamination (85%) was higher than that seen in samples from lines supplying other instruments, though the small sample size precluded a proper comparison.

DISCUSSION

Our findings indicate that high levels of *Legionella* contamination of water may be encountered in dental operatories. The actual concentration of *Legionella* spp., however, varied from one dental operatory to another, consistent with the literature [Pankhurst *et al* 1990]. This

suggests that the microbial ecological conditions in dental lines may fluctuate and affect *Legionella* populations.

Compared with contamination of domestic potable waters, the intensity of the *Legionella* contamination of dental-unit water samples was at least an order of magnitude higher. The levels seen in this study in both the dental water and the potable water samples were higher than have been recorded for each previously; this may be a reflection of the higher performance characteristics of the PCR-DNA detection system relative to those of conventional cultivation procedures [Mahbubani *et al* 1990, Stout *et al* 1992]. Given this greater sensitivity of the PCR-DNA probe detection method [Mahbubani *et al* 1990], it appears that low counts (in the <10,000/ml range) may prove clinically significant only for individuals whose exposure to the aerosol occurs over prolonged periods (for example, for dental office personnel), or immunocompromised individuals [Winn *et al* 1988].

The growth of *Legionella* within amoebae [Anand *et al* 1983, Breiman *et al* 1990, Fields *et al* 1990, Fields *et al* 1993, Fields *et al* 1984, Harf *et al* 1987, Henke *et al* 1986, Newsome *et al* 1985, Rowbotham *et al* 1986, Tyndall *et al* 1982, Wadowsky *et al* 1988] is one of the reasons that

detection of the bacteria by culture is extremely difficult. While DFA allows visualization of the intraprotozoan *Legionella*, the effects of this sequestration on PCR-gene probe detection are not known. That both PCR and DFA give similar prevalence and intensity results suggests that effects on PCR are minimal [Atlas *et al* 1995].

The higher concentrations of *Legionella* detected by PCR than by viable-plate-count methods also raise the question as to whether nonviable *Legionella* species that have no clinical significance are detected by the PCR method. Relatively short amplicons, as in the EnviroAmp detection system, can be used to detect nonviable *Legionella spp.* [McCarty *et al* 1993]. Detection of *Legionella spp.* by direct fluorescent-antibody detection gave results similar to those by PCR detection; both methods gave results that are higher than those obtained by viable-culture methods, and both could detect nonviable rather than exclusively viable *Legionella* [Atlas *et al* 1995]. The viable-culture methods for *Legionella* detection, however, often fail, and the Centers for Disease Control and Prevention has turned to PCR for epidemiological investigations of Legionnaires' disease and Pontiac fever [Miller *et al* 1993]. Furthermore, viable nonculturable *Legionella spp.* have been shown to be capable of causing pulmonary legionellosis [Reingold *et al* 1984];

exposure to high concentrations of viable nonculturable *Legionella spp.* may also be an important cause of Pontiac fever [Miller *et al* 1993].

Legionella spp., the causative agents of Legionnaires' disease, Pontiac Fever, and a variety of local infections, are ubiquitous in aquatic environments, [Dondero *et al* 1980, Fliermans *et al* 1981, Stout *et al* 1992, McDade *et al* 1977, Winn *et al* 1988]. Infection is most often acquired through the inhalation of aerosols containing high levels of *Legionella* spp. by susceptible individuals; though ingestion, direct inoculation, and irrigation of surgical wounds by contaminated water have also been implicated [Dondero *et al* 1980, Fliermans *et al* 1981, Stout *et al* 1992]. Exposure to low numbers of *Legionella* organisms is generally not viewed as a health risk for immunocompetent individuals [Winn *et al* 1988]. While air-conditioning cooling towers have been considered the most likely source of heavy exposure, potable water supplies, hospital showerheads, and even vegetable moisturizers in produce markets have been implicated in past outbreaks of *Legionella* infections [Bolin *et al* 1985, Dondero *et al* 1980, LaMaine *et al* 1990, Stout *et al* 1992, Winn *et al* 1988].

Dental-unit water, especially when aerosolized, is a potential source of exposure to *Legionella* species [Abel *et al* 1971]. Previous studies utilizing traditional culturing approaches have found that *Legionella* species could only be cultured sporadically, with between 9% and 40% of the dental-unit and potable water samples examined yielded culturable *Legionella* spp. [Borneff *et al* 1986, Reinthaler *et al* 1986].

In spite of this sporadic culture detection, serological surveillance of a dental staff and a control group revealed elevated seropositivity among dental personnel. In Dresden, Germany, an elevated level of anti-*L. pneumophila* SG6 antibodies was found among dentists [Luck *et al* 1993], particularly among those working in dental operatories where L. *pneumophila* serogroup 6 (SG6) was isolated. Similar results were obtained in a study of dentists, dental assistants, and dental technicians in Austria [Reinthaler *et al* 1988]. In the latter study, there was a strong positive correlation between seropositive individuals and the degree of exposure to aerosols from high-speed drills and dental syringes. Thirty-four percent of the sample group was seropositive for *L. pneumophila,* compared with only 5% testing positive in a control group of nonmedical workers. Of the 36 positive serum samples, 13 (36%) reacted with SG6,

12 (33%) with SG1, 12 (33%) with SG5, and 3 (8%) with SG4; 8 samples were positive for antibodies to other *Legionella* species. Among the sample population, dentists had the highest prevalence (50%) of L. *pneumophila* antibodies, followed by dental assistants (38%) and technicians (20%). In another study, 20% of the students and employees at a dental clinic in West Virginia were seropositive for *Legionella* antibodies [Fotos *et al* 1985]. These results suggest that dental personnel are at an increased risk of *Legionella* exposure.

These findings of high-prevalence/low-intensity contamination of *Legionella* in potable water supplies was somewhat unexpected. The concentrations seen are higher than those permitted by EPA regulation [Hansen 1988] or those seen in previous studies based on the viable culture technique [Marrie *et al* 1994, Ta *et al* 1995]. This finding reflects the increased sensitivity of the PCR technique [Mahbubani *et al* 1990, Stout *et al* 1992]. The low intensity seen in the majority of these samples (in the <100/ml range) is probably clinically insignificant [Atlas *et al* 1995, Winn *et al* 1988], although more data on environmental sources, their degree of contamination, and links to disease occurrences now need to be calculated for the new, more sensitive technique. It would seem appropriate for

regulatory agencies to reevaluate the acceptable limits for *Legionella* contamination of potable water, basing their rules on clinically significant intensities, taking into account the method of detection employed.

The high intensity of contamination with *Legionella,* well above the 10,000/mL level, in dental-unit water samples as compared to potable water samples may be a reflection of the rich microbial biofilms commonly present along the runs of the fine-bore dental water tubing [Williams *et al* 1993]. In previous studies, *Legionella* organisms appeared to be growing within biofilm in the dental-unit water supply, often within protozoa [Pankhurst *et al* 1990, Michel *et al* 1989]. Amoebae have been seen frequently in dental-unit biofilms [Michel *et al* 1989, Williams *et al* 1993].

In light of the results reported here, it is surprising that no definitive clinical associations between dentistry and legionellosis have thus far emerged. There were no related cases of human infection detected in two studies at dental institutions in Britain where *Legionella* spp. were isolated from dental-unit water [Oppenheim *et al* 1987, Pankhurst *et al* 1990]. There may be several reasons for the lack of association of dental-unit water and occurrences of Legionnaires' disease.

Nonpneumonic legionellosis of the Pontiac fever type may occur in dental personnel or their patients and cause seroconversion but may be indistinguishable clinically from other flu-like episodes experienced by the general population. *Legionella* spp. vary with regard to their virulence. Most *Legionella* spp. detected in this study were not *L. pneumophila;* thus they would be expected to be less virulent than *L. pneumophila* [Winn *et al* 1988] because they lack the macrophage infectivity potentiator gene and other factors of *L. pneumophila* which are involved in cell invasion and subsequent intracellular growth. On the basis of guinea pig infectivity studies, it has been suggested that even the *L. pneumophila* strains commonly present in dental-unit water may have limited invasive capacity [Luck *et al* 1993]. While *L. pneumophila* was more prevalent in dental water than in potable water supplies, concentrations of *L. pneumophila* generally were much lower than those of the other *Legionella* species, and *L. pneumophila* was detected at levels below those normally considered to present a health risk for immunocompetent individuals [Winn *et al* 1988].

Interestingly, the sources of most cases of community-acquired pulmonary legionellosis are never identified [Stout

et al 1992]. This is because the majority occur as sporadic cases rather than as outbreaks, and no epidemiological investigation into the source is attempted. In order to make a definitive connection between a source and clinical disease, specific comparisons of isolates must be made between the organisms cultured from the water and the clinical isolates (when they are obtained). One method of making this comparison employing a modern molecular biology technique is reported in a subsequent chapter of this dissertation.

Unfortunately, cultures are not generally obtained from clinical specimens of sporadic cases. The diagnosis of legionellosis is typically made when a patient presents with an atypical pneumonia and organisms are seen on indirect fluorescent antibody staining of sputum, positive urine antigen test, or a rise in titer is seen on serial serology [Henkel *et al* 1995]. When a high index of clinical suspicion of legionellosis surrounds a case even in the absence of demonstrable organisms, empiric antibiotic treatment is begun following initial serology. In this author's limited clinical experience, when the patient recovers, follow-up titers are often not obtained. As a result, the total number of cases of Legionnaire's disease may be significantly under-reported. Given the findings of

Legionella contamination of dental water, the potential implication of dental exposure may represent a previously unrecognized but important element of the medical history of a proportion of the cases for which a source has not been found.

Regardless of the lack of specific clinical association, exploration of possible preventive measures against *Legionella* spp. and other opportunistic waterborne pathogens [Williams *et al* 1993] in the dental health care setting would be prudent. Preventive strategies increasingly adopted by hospitals faced with nosocomial legionellosis outbreaks have ranged from chemical disinfection to steam sterilization to rid water lines of contaminating biofilm. Hyperchlorination and charcoal filtration have been shown to be ineffective in controlling *Legionella* spp. contamination of dental lines [Pankhurst *et al* 1990], but the application of other measures such as those based on microfiltration, germicidal flushes, or fully autoclavable fluid reservoirs and connecting tubings would clearly be appropriate.

Chapter 3

Contamination of Institutional Dental Unit Water by Heterotrophic Bacteria and *Legionella*

INTRODUCTION

Microbiological profiles of dental unit water (DUW) in institutional facilities at university dental schools and in the dental clinics of large hospitals have been featured in a number of reports from North America and Europe [Abel *et al* 1971, Fitzgibbon *et al* 1984, Fotos *et al* 1985, Reinthaler *et al* 1986]. Contamination with *Legionella* species and the resulting occupational exposure of personnel have been identified as potential problems as discussed in chapter 2 [Atlas *et al* 1995]. The quality of water in these institutional settings may be affected by the deterioration that often characterizes plumbing systems in large aging buildings [Bezanson *et al* 1992, Hart *et al* 1991, Memish *et al* 1992]. Declining water quality has been shown to result in chronically contaminated water supplies in many medical facilities, and there is correspondingly a high frequency of

nosocomially acquired water-borne infections in hospitalized patients, including legionellosis (Legionnaires' Disease) and *Mycobacterium avium* infections [Hart *et al* 1991, Slosarek *et al* 1994, Sniadack *et al* 1993].

Legionella, especially *L. pneumophila,* the species responsible for the original outbreak of acute pneumonia amongst conventioneering legionnaires that gave the disease its name [Fraser *et al* 1977] is also recognized as the fourth most common cause of community-acquired (i.e. non-nosocomial) pneumonia [Mandel 1995, Ostergaard *et al* 1993]. The intensity of *Legionella* contamination in DUW samples drawn predominantly from private dental offices has been shown to be much higher than in domestic potable water sources [Atlas *et al* 1995], although the relationship between these degrees of contamination and the occurrence of clinical legionellosis remains unknown.

This chapter reports the results of a study which encompassed several important aspects of *Legionella* contamination of dental units. It is based primarily upon assembled data on the extent of microbial contamination, including *Legionella,* in dental unit water samples from fourteen institutions: nine in the USA and five overseas.

First, the presence of *Legionella* was detected and semi-quantitatively assessed using a PCR amplification procedure which employs genus and species specific molecular probes [Mahbubani *et al* 1990]. Second, primary isolation and identification of *Legionella* was undertaken on some samples, employing the direct fluorescent antibody (DFA) immunostaining and repetitive-element PCR-amplification (rep-PCR) product electrophoresis to identify organisms. This part of the study provided an opportunity to compare the sensitivity and utility of these approaches to the evaluation of *Legionella* in water samples. Third, the study permitted an exploration of certain critical factors that influence the outcome of the widely used *Legionella*-PCR environmental detection kit which is commercially available. Finally, the molecular detection method was used to investigate the potential for detection of *Legionella* in aerosols created around dental operatories during the use of powered dental instruments that use coolant water.

The above techniques and the experiences gained in their use were subsequently applied to a series of water samples from the offices of a practicing dentist who had suffered an episode of subacute pneumonia believed to be legionellosis,

possibly associated with the high numbers of organisms in the water throughout his dental equipment.

The results are discussed in terms of their significance to the emerging picture of *Legionella* contamination in dental facilities and the potential health importance of these findings. The advantages and shortcomings of molecular biological approaches to detection of environmental pathogens like *Legionella* are also discussed.

MATERIALS AND METHODS

Water samples

A total of 241 dental unit water samples were processed in the course of this study. Samples in this study, as opposed to those of the studies reported in the preceding chapters, came exclusively from institutional dental clinics, i.e. those affiliated with a university or major medical center. Such clinics, in our experience, are more likely to follow strict infection-control and disinfection protocols than are private practices. As such, one might expect contamination levels to be lower than those seen in the samples from mixed populations of clinics already discussed. Conversely, these institutional clinics are more likely to be in older buildings, and use equipment that has been in service for a

longer period of time; these features may result in *higher* contamination levels than those reported in the earlier chapters. Institutional samples were furnished through the cooperation of personnel at the following sites; University of Minnesota Dental School, Minneapolis MN, University of Oregon, Dental School, Portland, OR, Forsyth School of Dental Hygiene, Northeastern University, Boston, MA, Pierce College School of Dental Hygiene, Tacoma , WA, Lansing Community College School of Dental Hygiene, Lansing, MI, Sacramento Community College, School of Dental Hygiene, Sacramento, CA, Harborview Hospital Dental Clinic, Seattle, WA, Children's Hospital Foundation Pediatric Dentistry Clinic, Cincinnati, OH, and Jackson State Prison Dental Clinic, Jackson, MI. Overseas samples were collected at the Dental Clinic of the University of Zulia, Venezuela, and at the dental clinics associated with several of the major hospitals in Beijing, China, including the Beijing Hospital, the Xian Hospital and the China-Japan Friendship Hospital.

Samples collected by cooperators in the U.S. were handled as described previously [Williams *et al* 1993] and shipped via overnight carrier using ice-packs to keep the water cool in transit. Samples from overseas sites were hand-carried by colleagues (Dr. Neuro Guanipa, University of Zulia, and Dr. Ziao Tan Qiao , University of Beijing) on ice in coolers

and processed for heterotrophic plate counts as soon as possible on arrival, typically within 12-24 hours. Samples from the local site in Lansing were hand carried to the MSU laboratory on ice and processed the same day. Dental unit water from the offices of the private practitioner in San Francisco were collected by office staff approximately four months after the practitioner had been hospitalized for subacute pneumonia associated with a high titer of antibodies against *L. pneumophila*. These were handled as specified above for the institutional samples and shipped overnight to MSU for processing.

Water from a wide variety of dental hand instruments water lines was studied, including several connected to so-called "self-contained" water systems which utilize a refillable bottle as the source of water rather than the municipal tap water. Some samples came from dental lines subjected to routines of chemical disinfectant flushing, varying from a few minutes of exposure to regular soaking for up to two hours. While most samples were obtained via convenience sampling throughout the work day of the operatory (so as to be representative of the water delivered to the patients' mouths), some of the samples were collected at specific intervals after flushing protocols as detailed in the results section below.

Aerosol samples

Aerosols were collected using an Anderson sampling vacuum pump (Anderson Co., Atlanta, GA) and AGI-30 glass impingers (Ace Glass Co., Vineland, NJ) containing sterile water, as described by Trudeau [Trudeau *et al* 1994]. In this procedure, a calibrated vacuum generator draws a measured volume of air containing the aerosol into glass vessels (impingers) set up a specific distance from the aerosol source. Over the course of a known time interval, aerosolized particles in the sample strike the surface of the sterile collection fluid within the impinger and are retained. Following collection, the fluid is removed and cultured or processed for PCR. In this study, impingers were set up at a distance of 60 cm from the area of dental work in a patient's mouth and a total of 0.33 cubic meters of air were sampled for each of eight tested operatories: four from operatories using ultrasonic scaler lines and four from high speed handpiece lines. Samples were shipped on ice via overnight courier and processed for culture and PCR-based *Legionella* detection.

Heterotrophic bacteria

Heterotrophic plate counts (HPC) were performed according to the procedure described in chapter one. Briefly,

heterotrophic bacterial contamination of the samples was assessed by plating 100uL aliquots of serial dilutions of each sample onto R2A plates and incubating as previously described [Santiago *et al* 1994, Williams *et al* 1993]. Plates with >300 or <30 colony forming units (CFU) were considered too many, or too few to count, respectively, for statistical reasons [American Public Health Association 1985]. Every effort was made to avoid undue delays between collection and HPC determinations, since ideally these should be carried out as soon as possible after sample collection to avoid artifactual changes in bacterial numbers. Samples were routinely chilled, although this is known to alter the culturability of certain water organisms. Preliminary studies in our laboratory showed that changes in HPC numbers were not significant over the first week of storage at 4C, but that storage for longer periods leads to unacceptable declines.

Molecular Detection of *Legionella*

Each water sample was filtered through a Durapore filter to trap bacterial cells, and the trapped bacteria were lysed by treatment with EnviroAmp lysing reagent (Perkin Elmer-Roche Molecular Systems, Nutley, N.J.). An aliquot of the sample was transferred to a reaction vessel for amplification of the diagnostic gene sequences. The EnviroAmp detection kit

was used for PCR amplification and gene probe detection. This kit includes PCR buffer, *Taq* DNA polymerase, and biotinylated primers for amplification of a genus-specific region of the *Legionella* 5S rRNA gene (5'-poly-dT-GCGCCAATGATAGTGTG-3' and 5'-poly-dT-GCGCCGATGATAGTGTG-3') and of the *L. pneumophila mip* gene (5'-GCATTGGTGCCGATTTGG-3' and 5'-GCTTTGCCATCAAATCTTTCTGAA-3'/5'-GTTTTGCCATCAAATCTTTTTGAA-3'). A Quarterbath (Inotech Biosystems, Inc., Lansing, Mich.) thermal cycler was used for amplification with a 30-cycle program of 0.5 min at 95C for denaturation and 1 min at 63C for primer annealing and DNA extension.

The PCR-amplified 5S rRNA and *mip* DNA sequences were detected by reverse dot blot strip analysis with an immobilized probe. Specific probes complementary to internal sequences of the amplified regions are immobilized on nylon membrane strips in the detection kit. Biotinylated PCR products generated by amplification with biotinylated primers are specifically hybridized to the immobilized probes. After stringent washing of the strips, the presence of hybridized biotinylated PCR products are detected by incubating the strips with a streptavidin-horseradish peroxidase conjugate, washing, and adding the substrate for horseradish peroxidase. A blue dot appearing on the nylon membrane indicates the presence of bound PCR product.

An internal positive control (IPC) is included as a means for detecting poor amplification or hybridization. The IPC is a synthetic DNA sequence that is coamplified by the primers for the *mip* gene, and its template is included in the EnviroAmp PCR mixture. When the IPC fails to yield the positive blue dot, it indicates poor amplification such as may be caused by the presence of inhibitors of PCR in the environmental specimen. In an effort to overcome this inhibition, aliquots of the samples were diluted 1:10 and processed in parallel with the undiluted aliquots. As a result of the pattern of inhibition experienced in processing these samples, duplicates were processed independently for comparative purposes by Dr. Claudia Thio at Perkin Elmer Cetus PCR Laboratories in Palo Alto, CA, and by Dr. C. Paszko-Kolva at Advanced Technology Laboratories in Alta Loma, CA.

The positive control also provides a semiquantitative basis for estimating the number of *Legionella* cells in the sample. The IPC corresponds to 1,000 copies of the *mip* gene sequence prior to amplification, and thus the intensity of the sample hybridization signal can be graded on a scale to determine the relative number of *Legionella* cells.

An internal negative control is also included in the kit as a means of ensuring appropriate hybridization stringency. The positive control probe is perfectly complementary to a sequence in the IPC amplification product. The negative control probe has a 1-bp mismatch with this sequence. When the hybridization reactions are done correctly, the PCR product generated from amplification of the IPC will hybridize with the positive control probe but not with the negative control probe. The hybridization conditions have been optimized in this system to be stringent enough to allow detection of a 1-bp mismatch between the probe and a PCR product.

In this study as well as that reported in chapter 2, we found frequent, significant inhibition of the PCR amplification by unknown substances within the dental unit waters; thus, it was necessary to process an aliquot of such samples both directly and as a 1:10 dilution in an effort to overcome the inhibition. This modification of the protocol, as recommended by the kit manufacturer, was modestly effective as will be discussed later in this chapter.

Direct Fluorescent Antibody staining

The direct fluorescent antibody (DFA) procedure was employed to identify *Legionella* in sediments of certain samples to determine the comparability of DFA- and PCR-based techniques for the detection of these organisms in dental unit water. DFA was also used to specific identify isolates obtained using the primary isolation and selection procedure described above.

Sediments or suspensions of organisms from picked colonies were dispensed (approximately 10^8 cells/mL) in 10 microliter aliquots onto acid washed microscope slides and allowed to dry before acetone fixation for 10 minutes. Slides were stained using pooled FITC-conjugated polyclonal rabbit antisera provided by the Michigan Department of Public Health. Following staining at room temparature for 30 minutes, slides were rinsed in PBS and distilled water, air dried, and examined by fluorescence microscopy.

The above antisera have been prepared to discriminate based on two pools of antigenic characteristics (pool A: *L. pneumophila* serotypes, and pool B: non-*pneumophila Legionella* species). Once positive results were obtained with a pool reagent, antibody preparations of more restricted specificities were applied (pool A1, A2, A3

etc.). These pools consisted of pool A1 (specific for *L. pneumophila* SG2, SG3, SG4, SG12); pool A2 (*L. pneumophila* SG5, SG6, SG8, SG10, *L. lansingensis*); pool A3 (*L. pneumophila* SG7, SG9, SG11, SG13, SG14); pool B1 (*L. dumoffii, L. gormanii, L. longbeachae* SG1, *L. longbeachae* SG2, *L. jordanis, L. anisa, L. tucsonensis*); pool B2 (*L. bozemanii* SG1, *L. bozemanii* SG2, *L. feeleii* SG1, *L. feeleii* SG2, *L. micdadeii*), pool B3 (*L. birminghamensis, L. cincinnatiensis, L. lansingensis, L. maceachernii, L. sainthelensi* SG1, *L. sainthelensi* SG2); and pool B4 (*L. hackeliae* SG1, *L. hackeliae* SG2, *L. wadsworthii, L. oakridgensis*).

Speciation beyond that obtained using the above pools was not attempted. In this study identification as to specific serotypes within species was not considered necessary in order to accomplish a comparison of sensitivity and to a degree, specificity, between methods. Control tests with known reference serotypes of *Legionella* spp were provided by the Michigan Public Health Laboratory, and conjugated preimmunization sera from the rabbits offered a negative control for the extent of non-specific binding with these reagents with known standards and with test samples.

Legionella cultivation

Primary isolation of *Legionella* was undertaken from certain sample collections following the procedures described by Yu *et al* [1987]. Water samples were centrifuged at 5000G for 30 minutes and the supernatant discarded. Sediments were resuspended in 200 uL KCl-HCl buffer, pH 2.0, and incubated at room temperature (25C) for 30 minutes after which they were neutralized with 0.1N NaOH. A one hundred microliter aliquot of each sample was plated on DGVP supplemented BCYE agar plates and incubated at 35C for up to 14 days, following established protocols [Centers for Disease Control and Prevention 1992].

Individual colonies that grew on the DGVP-BCYE were selected and suspended in 0.5mL sterile water, and 100 uL was plated on unsupplemented BCYE and on sheep blood agar (SBA). Those colonies which grew on BCYE but not SBA were used in these studies. Isolates of *L. pneumophila* SG1 provided by the Michigan Department of Public Health were used as controls. Isolates were maintained on BCYE slants under the same culture conditions.

rep-PCR fingerprinting of _Legionella_

Repetitive PCR fingerprinting of *Legionella* employed an adaptation of the methods used Verslovic and others

[Georghiou *et al* 1994, vanBelkum *et al* 1993, Verslovic *et al* 1994, Woods *et al* 1993]. Briefly, colonies were scraped from the BCYE plate (described above) and washed in 1M NaCl, then suspended in sterile ultrapure water. A 25 uL aliquot of this suspension was removed and subjected to repeated freeze-thaw cycles to lyse a portion of the cells.

Twenty-five microliters was then placed in a 0.5 mL thin-walled reaction tube with 50pmol each of two opposing primers. These included either ERIC2 (**5'-AAGTAAGTGACT GGGGTGAGCG-3'**) and BG2 (**5'-TACATTCGAGGACCCCTAAGTG-3'**) [vanBelkum *et al* 1993] or REP1R-Dt (**3'-CGGNCTACNGCNGCCNIII-5'**) and REP2-Dt (**3'-CATCCGGNCTATTCNGCN-5'**) [Georghiou *et al* 1994]. In addition, 1.25mM of dATP, dTTP, dGTP, and dCTP, 2U of *Taq* polymerase, and 10% DMSO were present in each tube. Total reaction volume was 25uL in a PCR buffer (5x buffer included 83mM sodium acetate, 335mM TRIS-HCl, 33.5mM $NaCl_2$, 33.5uM EDTA, 150mM β-mercaptoethanol, 850ug/mL bovine serum albumin; pH 8.8).

Amplification proceded with an initial 7 minute denaturation at 95C followed by 30 cycles of 30 seconds at 90C, 1 minute at 52C, and 8 minutes at 65C in a Perkin Elmer Cetus thermocycler.

Following amplification, products were stored at -20C until they were electrophoresed on a 1% agarose gel.

RESULTS

Heterotrophic bacteria

The results of microbiological analysis of dental unit water samples from the U.S. institutions are shown in Figure 1. Heterotrophic plate counts for samples from institutions in Venezuela and China are shown in Figures 2 and 3, respectively. The range of bacterial concentrations was from <30 CFU/mL to 2.65×10^7 CFU/mL.

PCR *Legionella* detection

Prevalence and the semi-quantitative intensity assessment of *Legionella* contamination are illustrated in Table 1 for samples from the institutions evaluated. *Legionella pneumophila* was clearly not the preponderant organism detected in most samples, but in one case almost half of those samples testing positive for the *Legionella* probe were also positive for *L. pneumophila*.

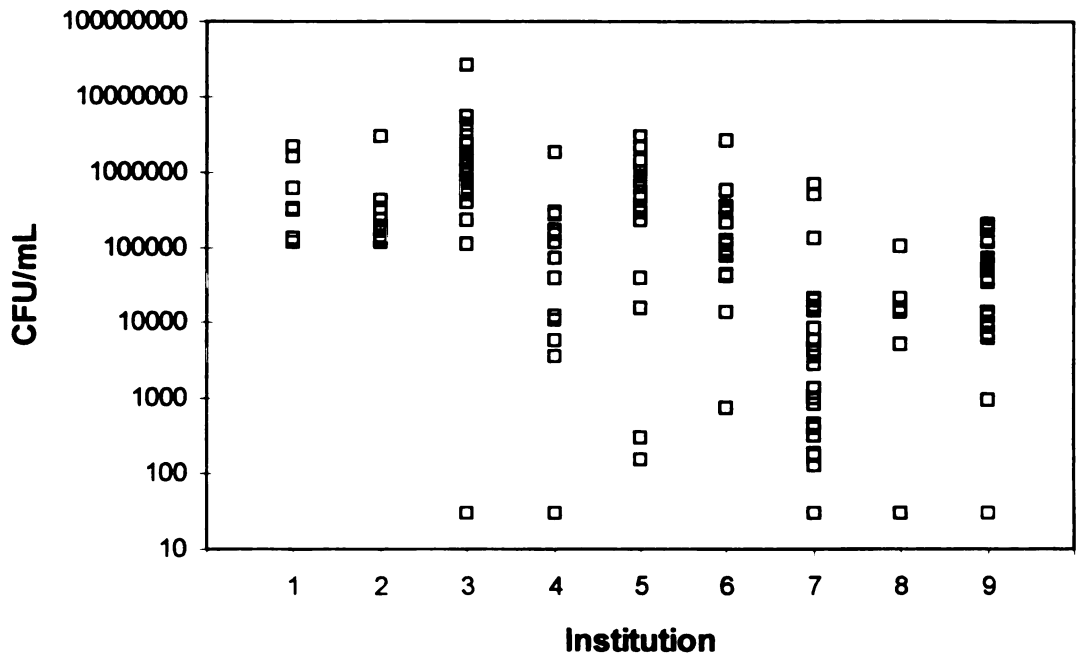


Figure 1. Scattergram depicting heterotrophic bacterial plate counts in USA institutional dental clinics. All samples from institution #8 are from waterlines connected to "self-contained" bottled water reservoirs where the lines are subject to routines of exposure to 5% bleach solutions, usually on a weekly basis. Gross bacterial contamination is evident in the majority of samples from every institution.

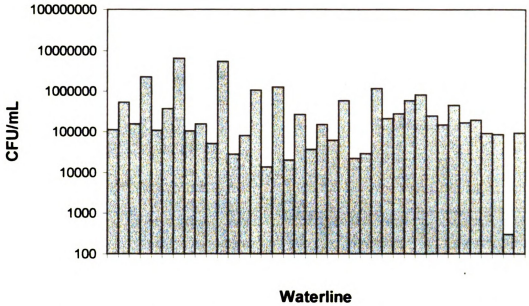


Figure 2. Histogram representing heterotrophic bacterial plate counts of dental unit water samples collected from an institutional dental clinic in Maracaibo, Venezuela. Each vertical bar represents the bacterial concentration in a sample from an individual dental unit waterline within the clinic. Gross contamination is evident in nearly every sample.

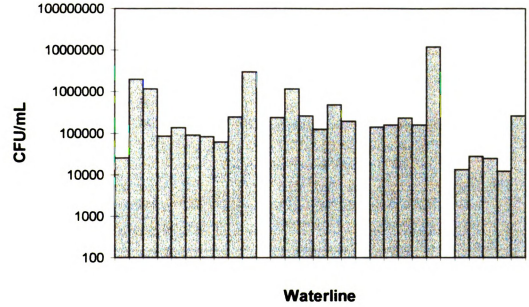


Figure 3. Histogram representing heterotrophic bacterial plate counts of dental unit water samples collected from four institutional dental clinics in Beijing, China. Each vertical bar represents the bacterial concentration in a sample from an individual dental unit waterline within the clinic. Gross contamination is evident in every sample.

Table 1. *Legionella* and *L. pneumophila* contamination of U.S. and international institutions. Source identification of U.S. samples reflects the identification numbers used in Figure 1. Institutions 5, 9, and two of the Chinese institutions were not assayed for *Legionella* contamination.

Source of the sample	n	# of samples with 10-10^3 *Legionella* organisms	# of samples with >10^3 *Legionella* organisms	# of samples with *L. pneumophila* detectable
US1	6	3	3	0
US2	5	1	4	0
US3	28	15	2	0
US4	19	3	15	8
US6	13	6	7	1
US7	20	15	0	2
US8	12	0	0	0
China 2	5	2	0	0
China 4	5	4	1	0
Venezuela	15	1	14	0

Relationship between heterotrophs and *Legionella*

A comparison of the heterotrophic bacteria concentrations for individual lines from one of the domestic institutions and the *Legionella* concentrations (based on PCR/DNA probe tests) from the corresponding lines are shown in Figure 4. There is no demonstrable relationship between heterotrophic bacterial and *Legionella* contamination intensities. Some samples with high levels of heterotrophs had low levels of *Legionella,* and *vice versa*. This was true for all four of the USA institutions for which this comparison was made, as well as for the Chinese and Venezuelan institutions. Thus, neither the likelihood of *Legionella* contamination nor its intensity in an individual sample could be predicted based upon its heterotrophic bacterial contamination level.

EnviroAmp PCR interference

The EnviroAmp kit includes a positive control for each sample that is standardized such that both qualitative and semi-quantitative conclusions may be reached regarding the intensity of the contamination. In this study as well as that in chapter 2, PCR amplification was inhibited by unknown substances within the majority of the dental unit water samples; in validating the EnviroAmp^{tm} kit, it was discovered that high levels of various organic materials

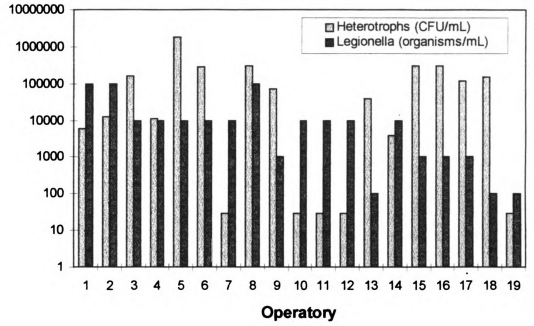


Figure 4. Histogram illustrating the lack of relationship between heterotrophic plate counts and *Legionella* concentrations in dental unit water samples from Institution #4 from Figure 1. There is no demonstrable relationship between heterotrophic bacterial and *Legionella* contamination intensities. Neither the likelihood of *Legionella* contamination nor its intensity could be predicted based upon heterotrophic bacterial contamination level. Operatories 3, 15, 16, and 18 are from "self-contained" bottled water reservoir systems that are subject to weekly 5% bleach solution disinfection procedures. Samples 1, 2, 4, 9, 10, 11, 12, and 14 were also positive for *L. pneumophila*.

including humic acid can inhibit the amplification reaction (Perkin Elmer Cetus, personal communication). In this study, it became necessary to process an aliquot of each sample both directly and as a 1:10 dilution. Five distinct patterns were observed in the results of the reverse dot-blot. These are illustrated in Figure 5.

The first row of the figure illustrates the case of complete inhibition of the amplification in which the positive control ("+") was not amplified. Inhibition in this sample was obvious, and necessitated a repeat of the analysis using a 1:10 dilution. The result was amplification and detection of *Legionella* DNA. This phenomenon occurred so frequently in dental unit water that each sample had to be processed as neat and a 1:10 dilution. In doing so, several additional variations surfaced between results on diluted and undiluted samples. The second pattern, seen in row two of Figure 5, illustrates a decrease in intensity of the signal from the DNA (as would be expected following the log decrease in sample-derived template produced by the dilution). Such is the case in the absence of inhibition by components of the water sample. However, row three illustrates a strip in which the positive control indicated a successful amplification, but in the 1:10 dilution it is clear that there was enough inhibition present to mask the low-level *Legionella* signal, while not affecting the visibility of the positive control. Similar effects are seen in the fourth row, in which the *L. pneumophila* species-specific signal became apparent only on dilution; *Legionella* spp. and control were amplified sufficiently in both preparations of this sample. Since dilution of samples appeared advantageous, the question arose whether it would be

possible to evaluate each sample based solely on the 1:10 dilution. In row five a strip is illustrated which indicated that the use of the 1:10 dilution would not be appropriate. Here the weak signals may have dropped below detectable levels with the diluted sample. This may well happen for the *Legionella* signal in samples having high levels of inhibitory factors. This would likely result in failure to detect low levels of contamination in the neat preparation (secondary to inhibition) and in the 1:10 preparation (secondary to dilution).

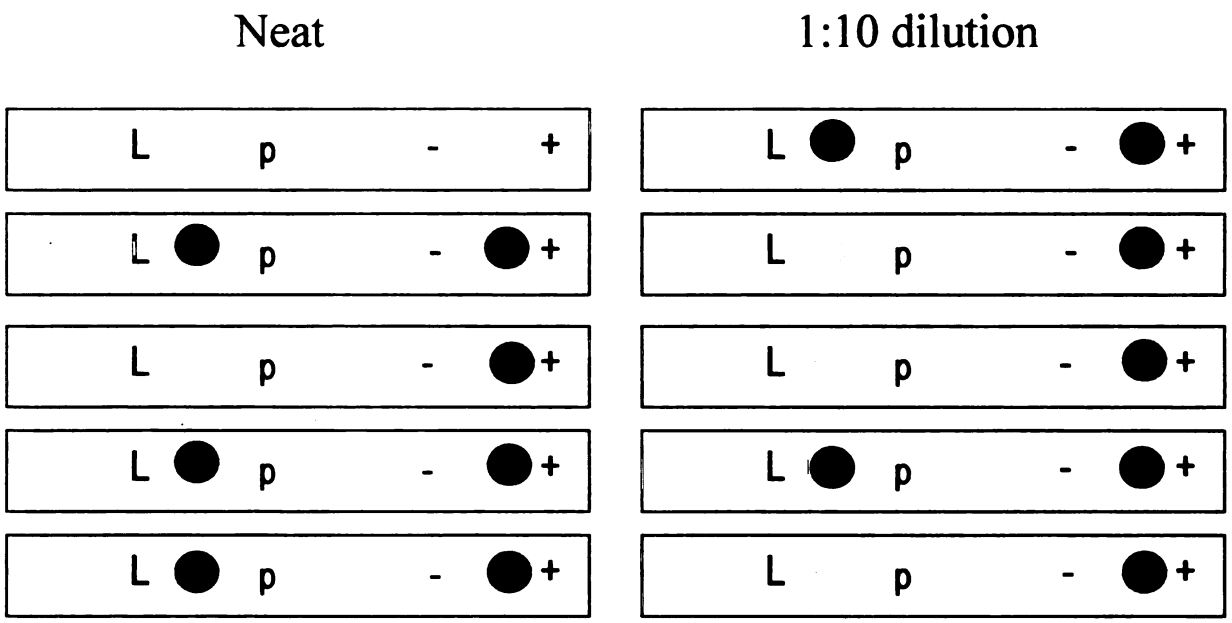


Figure 5. Schematic representation of EnviroAmp[tm] reverse dot-blots demonstrating the PCR inhibition observed in dental water samples. A dot at "L" indicates a positive signal for *Legionella*, at "p" indicates a positive signal for *L. pneumophila*, and "-" and "+" represent the negative and positive controls, respectively. The first row demonstrates inhibition of the PCR reaction, as evidenced by lack of amplification in the positive control in the neat preparation, overcome in the 1:10 dilution. Row 2 shows the results of dilution in the absence of inhibition. Rows 3-4 show partial inhibition, where the positive control amplifies in the neat, but the *Legionella* and *L. pneumophila* signals are seen only in the dilution. The final row shows that very weak signals drop below the limit of sensitivity of the test when the sample is diluted, precluding processing all samples exclusively at the dilution without also looking at the neat preparation. See text for further discussion.

Recognizing the constraints of the labeled probe technique revealed by these comparisons, all DNA probe results were recorded only after each sample had been examined both neat and at 1:10 dilutions in the test.

Aliquots of the samples used to generate the series of result shown in Figure 5 were sent, blinded, to two collaborators who were expert in the use of EnviroAmp^{tm} and other PCR modalities. Their results, shown in Table 2, suggested that variables produced by inhibitory factors confounded the interlab comparisons and were not easily overcome by either dilution or protocol modifications introduced by the manufacturer. At Perkin Elmer Cetus, the samples were processed both neat and at 1:10 dilution, and in each case the samples were processed in both the standard way, and using a modification to remove nonspecific inhibitors. This consisted of the addition of 3% bovine serum albumin (BSA) to the preparation immediately prior to amplification. This modification permitted detection of a *L. pneumophila* signal in one neat sample that had not been evident under standard sample preparation conditions. One other sample gave interpretable results (no nonspecific inhibition)only using the 1:10 dilution with 3% BSA, despite

having been interpretable at MSU at the 1:10 dilution using the standard preparation. Perkin Elmer Cetus results using either the modified or unmodified protocols failed to detect seven *L. pneumophila*-positive signals detected by both Advance Technology Laboratory and MSU. Overall, agreement between MSU and Advance Technology was 70%, between MSU and Perkin Elmer Cetus was 40%, and between Advance Technology and Perkin Elmer Cetus was 60%.

A comparison of the sensitivity of the DFA and PCR techniques in detecting the presence of *Legionella* is shown in Table 3, using samples from Institution #4. It is clear that PCR tests positive more frequently than DFA, but there was a troublesome inconsistency in the extent to which *L. pneumophila* could be found. Samples were seen that were *L. pneumophila* positive in DFA but not in PCR/DNA tests, and *vice versa*. Concordance of results was high when these procedures were applied to isolates.

The ability of EnviroAmp^{tm} to contribute to studies of heterogeneity of *Legionella* populations in samples is limited to differentiating *L. pneumophila* from non-*pneumophila* species. DFA, on the other hand, is well suited for this purpose. In Table 4 the results are shown of the application of this procedure to the exploration of a set

of 12 non-*pneumophila* isolates (i.e., negative by both PCR/DNA and DFA reagents for *pneumophila* specificity)

Table 2. Comparison of PCR-based *Legionella* detection in three laboratories. Fifteen water samples were processed by the three separate laboratories which were blinded to the results obtained at the other laboratories. Results noted as "inhibited" indicate that neither positive nor negative controls were amplified in any preparation. Interlaboratory agreement ranged from 7 to 40%.

Sample	MSU	Perkin Elmer Cetus	Advanced Technology
1	L+p+	L+p-	L+p+
2	L+p+	L+p-	L+p+
4	L+p+	L+p-	L+p-
7	inhibited	L+p-	L+p-
9	L+p+	L+p-	L+p+
10	L+p+	L+p-	L+p-
12	L+p+	inhibited	inhibited
13	L+p-	L+p-	L+p-
14	L+p+	L+p-	L+p+
15	L+p+	(spilled)	L+p+

Table 3. Comparison of DFA and PCR/DNA probe methods for detecting *Legionella* and *L. pneumophila* in sediment of samples from dental unit waterlines (n=19). Although the overall prevalence of *L. pneumophila* contamination was similar with each technique, there was not close agreement between the techniques for the individual samples.

	Legionella	***L. pneumophila***
DFA	11	7*
PCR	19	8**

*Five samples positive for *L. pneumophila* by DFA were negative by PCR.

** Six samples positive for *L. pneumophila* by PCR were negative by DFA.

derived from dental unit water samples collected at institution #6. Reactivities with the distinct pools of antisera to control cultures of non-*pneumophila* species (all of which had been isolated from clinical cases in Michigan) showed that the the group was heterogenous, with the preponderance of reactivities being to antigens in pool B2.

Legionella pneumophila was detected in the sediments of several of the samples from institution #6 with the PCR/DNA probe procedure but not the viable culture method. PCR tests of aerosol samples from operatories in this clinic were positive for the *L. pneumophila*. Altogether, three of eight aerosols sampled were *Legionella* positive by PCR, one from an operatory where an ultrasonic scaler was used (the

water samples from this line had been consistently positive for *L. pneumophila* by PCR) and two from operatories where highspeed handpieces were being used (water samples from both the highspeed handpiece lines had been positive for *Legionella* by PCR). Semiquantitiation of the DNA probe signals indicated that there were in excess of 100 *Legionella* organisms per cubic meter of aerosol collected in one case.

In another institutional setting (#4) where *L. pneumophila* appears to have a substantial presence in the dental waterlines via PCR, DFA serogroup-specific antibodies were used to type organisms seen in sediments. The results are shown in Table 5. No reactions were seen with antisera to SG1, the most common clinical isolate in the U.S. There were, however, reactions with SG6 and SG10, the next most important subtypes of *L. pneumophila*. Reactions with other serotypes were common, with some samples testing strongly positive with a variety of serotype-specific antibody conjugates. Fluorescent-stained organisms in sediments were occasionally seen in clusters, suggesting intracytoplasmic localization within the amoebae which were commonly present in these samples.

Table 4. Heterogeneity of DUW non-*pneumophila Legionella* isolates. DFA testing of dental unit water isolates of *Legionella* (non-*pneumophila*) from three instrument lines from institution #6. In 42% of the samples, multiple species of *Legionella* are detectable, demonstrating the complex heterogeneity of the DUWL *Legionella* populations. Reactivities of isolates are shown with pools of antisera directed against *Legionella* species groups. See text for details. Reactivity is recorded from - to ++++.

SAMPLE SOURCE	B1	B2	B3	B4
Airwater syringe #11	-	++	-	-
Airwater syringe #11	-	+++	-	-
Airwater syringe #11	+	++	-	-
Airwater syringe #11	-	+++	-	-
Airwater syringe #11	+	++++	+	-
Airwater syringe #11	+/-	+++	-	-
Airwater syringe #11	-	++	-	-
Airwater syringe #11	+	+	-	-
Airwater syringe #11	-	+	-	-
Airwater syringe #13	+	-	-	-
Highspeed handpiece #11	+	+	-	-
Highspeed handpiece #11	+	-	-	-

Table 5. Heterogeneity of *L. pneumophila* in dental unit waterlines. DFA testing of dental unit water sediments from instruments at instituition #4. Each sample contains multiple serotypes of *L. pneumophila*, indicating complex, heterogeneous populations of *Legionella* within the dental waterlines. Reactivities of isolates are shown with *L. pneumophila* serotype-specific reagents. Intensities of contamination are recorded as - to ++++.

	WATER SAMPLE NUMBERS				
SEROTYPE	**8**	**15**	**16**	**17**	**18**
1	-	-	-	-	-
2	-	++	+++	+++	++
3	-	++++	+++	++++	++
4	-	-	-	-	-
5	++	-	-	-	-
6	++	-	-	-	-
7	+++	-	++++	-	-
8	++++	++++	+++	++	+++*
9	++	-	++++	-	-
10	+++	++++	-	-	++++*
11	+++	-	-	-	+++*
12	-	-	-	+++	-
13	++++	-	-	-	-
14	+++	-	++++	+++	-
LN3	++	++++	+++	++++	++++

*Fluorescing organisms arranged in clumps, possibly within protozoan cells within the sediment

rep-PCR fingerprinting of *Legionella*

Repetitive PCR fingerprinting revealed four distinct clonal subpopulations (Figure 6). For our purposes, nomenclature of the strains detected by repPCR consisted of arbitrarily assigning the strain the identification of the colony in which the specific pattern was identified, i.e. the pattern seen in colony #1 of water sample #1 was named "1-1", even though it is also the pattern of colony #2 of sample #3. Clone 1-1 was the most prevalent population isolated, representing 44% of the colonies isolated and present in over half of the samples from which organisms were isolated. In two of the samples, multiple distinct clonal subpopulations were identified. As these samples were also evaluated via DFA, a direct comparison of the DFA and rep-PCR results is possible, and shown in Table 6.

Application of methods to a clinical case

When the opportunity arose to apply *Legionella* detection techniques to water samples from the offices of a dentist who had experienced a clinical episode attributed to *L. pneumophila,* I was eager to apply the above techniques to an "*in vivo*" situation.

A 60 year old dentist in San Francisco experienced marked respiratory distress and was diagnosed with an acute

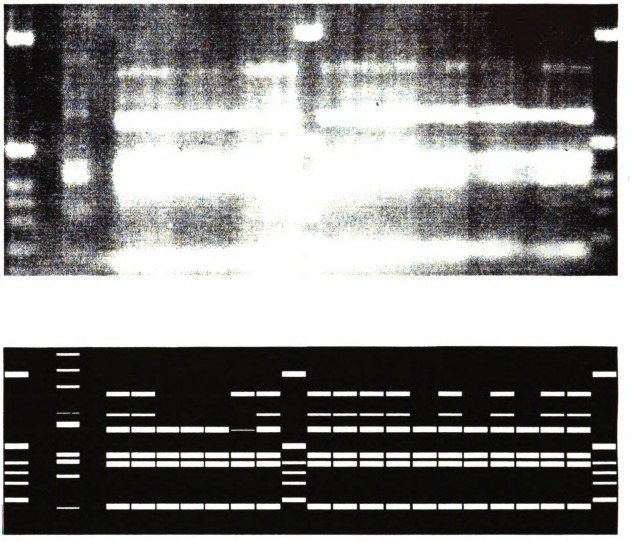


Figure 6. (a) photograph of, and (b) schematic representation of an agarose gel demonstrating the fingerprinting patterns observed between individual colonies within a single water sample, and between colonies from separate water samples. The top line of text under the schematic diagram denotes the strain identification (MDPH being the control *Legionella* culture provided by the Michigan Department of Public Health), and the bottom line of text indicates the water sample from which the colony was isolated. Nomenclature of the strains detected by repPCR consists of arbitrarily assigning it the identification of the colony in which the specific pattern was identified, i.e. the pattern seen in colony #1 of water sample #1 was named "1-1", even though it is also the pattern of colony #2 of sample #3. Clone 1-1 was the most prevalent population isolated, representing 44% of the colonies isolated and present in over half of the samples from which organisms were isolated. In two of the samples (3 and 7), multiple distinct clonal subpopulations were identified.

Table 6. Comparison of rep-PCR and DFA typing of *Legionella*. All samples contained multiple *Legionella* species, including multiple *L. pneumophila* serotypes, indicating a heterogeneous population of *Legionella* within DUWL as seen in previous tables. Despite the variety of *Legionella* detected by DFA, only a limited number of strains were culturable, and thus analysed by rep-PCR.

Sample Number	EnviroAmp PCR results* (organism/mL)	Number of colonies cultured	repPCR patterns present	DFA antisera pools reactive to sample
1	>1000/mL	1	1-1	A1, A2, B3
2	>1000/mL	1	1-1	A2, A3, B3, B4
3	>1000/mL	11	1-1, 3-1	A1, A2, A3, B1, B2, B3, B4
4	>1000/mL	3	3-1	A1, A2, A3, B2, B3, B4
5	>1000/mL	0	NA	A1, A2, A3, B2
6	>1000/mL	0	NA	A1, A2, A3
7	>1000/mL	2	1-1, 7-1	A1, A2, A3, B3
8	>1000/mL	0	NA	A1, A2, A3, B2, B3, B4

*Concentrations refer to non-*pneumophila* species, all samples were negative for *L. pneumophila* by PCR.

community-acquired pneumonia. He was empirically treated with rifampin and erythromycin and made a complete recovery. Serology drawn on presentation revealed a *L. pneumophila* titer of 1:512. No effort was made to isolate the etiological agent from his sputum, and no convalescent serum was obtained.

Sediments of water samples from his office were examined using the PCR/DNA probe approach and the results are shown in Table 7. High HPC counts were detected, as usual.

Table 7. HPC and PCR characterization of water samples from dental unit lines in the office of a dentist who experienced a probable *Legionella* infection. Each of the 16 samples had high levels of heterotrophic bacteria, and 100% were positive for *Legionella*, the majority at high levels. Over half were also contaminated by *L. pneumophila*.

SAMPLE NUMBER	CFU/mL	*Legionella*	*L. pneumophila*	*Legionella*/mL
1	6300	+	-	$>10^3$
2	71,000	+	+	$<10^3$
3	620,000	+	+	$<10^3$
4	97,000	+	-	$>10^3$
5	18,400	+	-	$>10^3$
6	148,000	+	-	$>10^3$
7	79,000	+	-	$>10^3$
8	124,000	+	-	$>10^3$
9	133,000	+	-	$>10^3$
10	181,000	+	-	$>10^3$
11	50,000	+	-	$>10^3$
12	98,000	+	-	$>10^3$
13	131,000	+	-	$>10^3$
14	300,000	+	-	$>10^3$
15	320,000	+	-	$>10^3$
16	38,000	+	-	$>10^3$

*Forty isolates of *Legionella* were obtained from these samples. *L. pneumophila* was present in samples 2, 4, 9, 10, 11, 12, 13, 14, and 15, based on PCR characterization of the isolates.

However, all of the 16 water samples from the dental lines in the office were *Legionella* positive, two with *L. pneumophila*. The majority of the samples contained well in excess of the 10^3 organisms per milliliter. Approximately forty *Legionella* isolates were obtained from primary culture of the samples. *L. pneumophila* were identified via PCR/DNA probe analysis in isolates from nine of the water samples.

Unfortunately, in that no clinical isolate was obtained, comparison of the rep-PCR fingerprints of the clinical and water sample isolates was not possible, precluding confirmation of the dental water as the proximate source of the infection.

DISCUSSION

These results permit a number of important conclusions to be drawn regarding the microbial contamination of dental unit waterlines and the issues surrounding the presence of *Legionella* in these lines in institutions. Overall, the data on HPC substantiate earlier findings from our laboratory on the extent of microbial contamination of coolant and irrigant water in dental equipment.

The data from samples collected in hospitals in Venezuela and China appear to be the first from these geographic areas to have been examined in this way, and they confirm the occurrence of heavy contamination in dental equipment of a variety of designs and origins.

Institutions, which might be considered more likely to have commitments to infection control practices (such as periodic hyperchlorination episodes) than would be likely in private offices, do not appear any less likely to be delivering microbially contaminated water to patients. On the contrary, some of the bacterial concentrations were extraordinarily high, although overall the HPC profiles from large clinics and private dental offices are very similar.

The high concentrations seen in samples from dental operatories that were equipped with "self-contained" water reservoirs routinely subjected to disinfectant flushes, were especially remarkable in the samples studied here. These results are a tribute to a) the readiness with which bacteria are able to get access to these supposedly "cleaner" self-contained systems, that are theoretically not subject to contamination by regrowth of municipal water organisms in the water supply, and b) the tenacity of the adherent microbes and their ability to maintain viable

biofilms in the face of periodic exposure to high concentrations of disinfectant chemicals. This finding has been reported recently by Williams and colleagues [H. Williams *et al* 1994] in a study of the use of self-contained water reservoirs by private dental practitioners. They found water samples issued from dental instruments in some of these offices to contain more than 5 x 10^6 organisms per milliliter , even though the dental practitioners believed that by using the reservoirs they had eliminated contamination. Indeed, most practitioners referred to their bottled water systems as "sterile water systems". In a survey of the practitioners [H. Williams *et al* 1994], a mere 15% followed protocols necessary to maintain acceptable contamination levels in the water in their equipment lines.

The high numbers of bacteria are due to the presence of well established and prolific biofilm layers inside the tubing, and their prodigious resistance to chemical dislodgment and disinfectant treatments are by now well known as a result of the published reports [Costerton *et al* 1987]. Biofilm organisms produce a glycocalyx slime matrix (known as extracellular polymeric substance, or EPS) which serves both as an anchor and as an anion exchange resin, capable of trapping nutrients based on their ionic charge, and preventing many biocides from reaching the resident

organisms. Biofilms form at many fluid-solid interfaces and are associated with pacemakers, prosthetic joints, other medical devices, and prolonged use of implanted catheters. Because of their resistance to antimicrobial agents, biofilm contamination of the surface of prostheses necessitates their removal and replacement [Costerton *et al* 1987, Gristina *et al* 1984, Gristina *et al* 1988, Kluge *et al* 1982, Marrie *et al* 1982, Nickel *et al* 1992, Peters *et al* 1981, Russell *et al* 1987].

It is certainly possible that the plumbing systems in some of the older educational institutions that furnished samples are contaminated with *Legionella* that were detected in the dental units [Bezanson *et al* 1992, Hart *et al* 1991, Memish *et al* 1992]. However, the amplification which leads to such high numbers of the organisms in dental unit water is most probably taking place within the long fine-bore hoses in the coolant and irrigant delivery systems. Conditions in these hoses, where prolonged stagnation is a characteristic of the use pattern favorable to *Legionella* growth, lead to the heterogeneous biofilms, especially rich in amoeba content, that can result in extremely high contamination in the output water. This has been observed in previous studies of the *Legionella* content of water in dental clinics associated with hospital facilities, especially in Europe [Michel *et al*

1984, Michel *et al* 1989, Williams *et al* 1993]. Seroconversion of staff and students to Legionella antigens have been reported under these circumstances, suggesting a degree of occupational exposure to these environmental organisms that is unique, there being no other published reports of occupational risks for exposure to *Legionella* to date.

Apart from the issue of *Legionella* and its detęction and significance, the numbers of heterotrophic organisms to which dental staff are exposed is rather high. At one of the sites used in this study, the concentration of microorganisms, largely Gram-negative pigmented colony formers in all probability [Williams *et al* 1993], averaged more than 10^6/mL. Some of these samples, collected as patients were being worked on, the samples had a mucoid appearance to the naked eye, probably because of the large amounts of carbohydrate substances produced in the biofilm and being continually sloughed off into the water during use. This process may be aggravated by the pulling and stretching of the tubing during dental instrument use for procedures [Santiago *et al* 1995].

Although no speciation of heterotrophs was attempted in this study, it is a reasonable that in addition to the biofilm

residents of an innocuous nature, there would also be opportunistic pathogens present and even some primary pathogens. They do not appear to bear any obvious relation to the presence or type of *Legionella* contamination, judging from the results summarized in Figure 4. *Legionella* were detected to similar degrees in both heavily contaminated and lightly contaminated samples, whether from high speed handpiece lines, scalers or air/water syringe lines. This is consistent with previous observations in the literature, although the recently published suggestion that certain equipment brands are more subject to *Legionella* proliferation than others is an interesting and potentially important development if confirmed [Challacombe *et al* 1995]. It suggests that specific features of equipment design may be contributory factors, and that by paying attention to them, the extent of contamination may be reduced. There was also no obvious relationship between HPC characteristics and the presence and numbers of *Legionella pneumophila*, as opposed to the genus *Legionella* only. The PCR/DNA probe results and the DFA analysis of the enormous heterogeneity suggest that, while the numbers of *Legionella* are exceedingly high, these organisms are usually mixtures of a number of species, and that *L. pneumophila*, when present, may be represented as a number of serotype variants. The absence of serogroup 1 type *L. pneumophila* in the limited

numbers of organisms specifically typed in one sample set here is reassuring since this is the agent associated with approximately 70% of clinical cases of legionellosis in the USA [Marston *et al* 1994]. Nevertheless, other serotypes and species are then left as the causes of almost a third of pneumonic legionellosis in the U.S.; these organisms are plentiful in the dental setting. In at least one fatal legionellosis case in a dentist, the causative organism, *L. dumoffi*, was obviously not in the LpSG1 category [Atlas *et al* 1995].

These observations about the occurrence of *Legionella* in dental unit water need now to be considered in the light of the findings reported here of the variables that affect the outcome of PCR/DNA detection techniques. In the past, the gold standard for *Legionella* detection in the environment had been primary isolation. This approach is at times cumbersome and unsuccessful in identifying sources of the organism [Atlas *et al* 1995, Mahbubani *et al* 1990, Stout *et al* 1992, Yamamoto *et al* 1993]. A recent investigation into recovery of organisms from water seeded with a known concentration of organisms using viable culture detection revealed that centrifugation concentration of organisms yielded recoveries of only 4 to 32%, while use of filter concentration exhibited a 50% recovery [Boulanger *et al*

1995]. For this reason tests such as DFA and the newly available DNA amplification procedures appear ideal. Interpretation can however be problematic, and although the procedures are finding more and more favor in the investigation of clinical episodes [Miller *et al* 1993], the results of this study make it clear that care must be taken to avoid implicating a water source as the source of clinical infection merely because *Legionella* are present. With the increased sensitivity of the modern techniques it is possible to detect clinically insignificant contamination.

The utility of the PCR/DNA test system is clear, and its superior sensitivity in detecting the presence of *Legionella* contamination over primary culture and DFA are becoming well known. The better detection rate of PCR over DFA when applied to dental unit samples seen here (Table 3) was not surprising, but the poor correlation with *L. pneumophila* detection between the two techniques was disappointing. Obviously, from the proven influence of unknown inhibitors on the outcome of PCR tests, there remain to be identified variables that are by no means insignificant. Whether or not there are interfering factors in the test outcome that are attributable to the presence of multiple species and serotypes of *Legionella* in one sample mixture is unclear,

but the findings with the samples from the dentist's office in San Francisco suggest such is the case: the *L. pneumophila* PCR detection rate on water sediments was low, but the primary culture results indicate that *pneumophila* organisms were present and isolatable from the majority of the samples.

The inconsistency of outcomes in PCR/DNA probe applications is very important for the adoption of PCR technology for environmental sampling. The inconsistencies that surfaced when identical samples were processed by three different laboratories suggest that the issue of inhibitors and their practical significance is still cloudy and needs further study. PCR as a means of environmental evaluation is certainly rapid in comparison to culture and isolation, but it is expensive and if samples need to be routinely examined at two dilutions, and perhaps by both standard and modified (BSA addition) protocols in order to get optimally interpretable results (i.e. minimal false negatives) the cost will be prohibitive. However, cost is not the most important factor. This kit is designed to detect *Legionella* in environmental water samples, samples likely to contain PCR inhibitors similar to those in dental water. Given the EPA's "zero tolerance" level for *Legionella* in potable water, the potential failure to detect lower levels of

contamination in the presence of inhibition of the PCR is unacceptable. It raises serious questions about the suitability of EnviroAmp *Legionella* or similar kits for use in other than investigational applications. There is a trend towards use of the same PCR primers in clinical sample processing and to date the problems of inhibition have not appeared [Matsiota-Bernard *et al* 1994]. The possibility that it might needs to be considered. It seems likely that the combination of culture/isolation and PCR-based characterization of *Legionella* isolates from different environments will be extremely useful in clarifying the epidemiology of *Legionella*, and in the process, modifications of the PCR protocol may be developed that will overcome the difficulties.

Certainly the convenience of the PCR approach is attractive and permits collection of data in circumstances that would otherwise not be likely to yield results. In the analysis of aerosols, for example, PCR techniques are now beginning to find acceptance [Alvarez *et al* 1995, Palmer *et al* 1995, Sawyer *et al* 1994], although the experience with *Legionella* to date appears to be very limited. The observation recorded here that *Legionella*, including *L. pneumophila*, was detectable in aerosols to which staff are occupationally exposed in a dental setting, has important implications.

Some of these relate to the practicalities of infection control in the dental office, and others revolve around legal and regulatory aspects of on-the-job exposure as discussed in chapter 2.

Of course the central issue of the clinical significance, if any, of the high levels of *Legionella* in the samples processed in this study will remain controversial, in the absence of prospective analysis of exposure rates and risk assessment. There were no related cases of human infection detected in two studies at dental institutions in Britain where *Legionella* spp. were isolated from dental-unit water [Oppenheim *et al* 1987, Pankhurst *et al* 1990]. There may be several reasons for the lack of association of dental-unit water and occurrences of Legionnaires' disease, including nonpneumonic or other unrecognized legionelloses, low virulence of the dental-water-derived organisms, or other situations as discussed in chapter 2.

Interestingly, the sources of most cases of community-acquired pulmonary legionellosis are never identified [Stout *et al* 1992]. This is because the majority occur as sporadic cases rather than as outbreaks, and no epidemiological investigation into the source is attempted. In order to make a definitive connection between a source and clinical

disease, specific comparisons of isolates must be made between the organisms cultured from the water and the clinical isolates.

One method of making this comparison is by employing a modern molecular biology fingerprinting technique such as rep-PCR. This technique exploits the naturally-occurring repetitive palindromic sequences interspersed throughout the genome of many prokaryotes. Using PCR primers based on these sequences, one may amplify numerous segments of the genome, with the fragments varying in number and size even between closely related strains [Verslovic *et al* 1994].

The results of the present study demonstrate that it is possible to obtain a molecular fingerprint of the organisms in dental water utilizing repetitive PCR technology. The application of this technique to compare operatory-derived and patient-derived isolates could prove useful in firmly establishing or disproving a clonal relationship between the isolates, though the natural fluctuations in *Legionella* populations and the existence of nonculturable strains will preclude its utility in all instances. Such an approach may be a useful tool in epidemiological studies of the relationship between dental operatory *Legionella* contamination and clinical legionellosis.

Unfortunately, cultures are not generally obtained from clinical specimens of sporadic cases. The diagnosis of legionellosis is typically made when a patient presents with an atypical pneumonia and organisms are seen on indirect fluorescent antibody staining of sputum, positive urine antigen test, or a rise in titer is seen on serial serology [Henkel *et al* 1995]. When a high index of clinical suspicion of legionellosis surrounds a case even in the absence of demonstrable organisms, empirical antibiotic treatment is begun following initial serology. In this author's limited clinical experience, when the patient recovers, follow-up titers are often not obtained. As a result, cases of Legionnaire's disease may be significantly under-reported. Given the findings of *Legionella* contamination of dental water, the potential implication of dental exposure may represent a previously unrecognized but important element of the medical history of the cases for which a source has not been found.

Over 30 *Legionella* species and more than 40 distinct *L. pneumophila* subtypes are described in the literature. Given the ubiquitous nature of these organisms in water and moist environments, it is common to isolate several different *Legionella* species/strains from a single plate of primary

culture [Barbaree *et al* 1993]. In spite of this, a previous study of dental clinic waters [Luck *et al* 1993] found that a stable, clonal population of *L. pneumophila* SG6 was present longitudinally, which suggested that Barbaree's observation might not hold true for dental water samples. Our findings are more consistent with the former report. DFA revealed multiple serogroups in all samples, and multiple species in all but one sample. Of these, we were able to culture and rep-PCR fingerprint four distinct clonal populations.

Eight colonies each were identified as clones 1-1 and 3-1, and in both cases these colonies were isolated from more than one sample. While these clones were the most prevalent strains among those isolated via culture, they may not represent the most prevalent subpopulations actually present in the original samples. There may be many other, more prevalent, strains present which are not as amenable to isolation via culture as are clones 1-1, 3-1, or even 7-1. Viable nonculturable *Legionella* spp., shown to be capable of causing pulmonary legionellosis [Reingold *et al* 1984] and Pontiac fever [Miller *et al* 1993], are detected by the PCR-gene probe method but not the viable culture method [Negron-Alvira *et al* 1989, Paszko-Kolva *et al* 1991, Stater *et al* 1987, Yamamoto *et al* 1993]. Our inability to culture, and thus fingerprint, the viable-nonculturable organisms

represents an important limitation to this study. We are unable to include them in any discussion of the genotypic clonality or heterogeneity of *Legionella* populations based on the current study.

The presence of two of the clones in multiple operatories within a single practice suggests the source for the organisms within the operatory lines originates upstream, possibly derived from the building plumbing or municipal water supply. Clones such as 7-1, which were only isolated from a single operatory, may represent strains of the practice-wide water system which have been selected by the ecosystem within the individual operatory, or patient-derived origins of the contamination. Although person-to-person transmission of *Legionella* has never been demonstrated, the organisms are present in sputum and could easily be introduced into the dental line via the suck-back phenomenon. However, given the close similarity of the banding patterns stains isolated in this study, especially compared to the divergence seen from the control strain, the former option is most likely the case in the practice on which this study is based. A comparison of subpopulations isolated longitudinally from these operatories to investigate the progress of genetic divergence of *Legionella*

clones within the individual ecosystems of each operatory is a topic worthy of further study.

The presence of *L. pneumophila* in dental water should not overshadow the potential contribution of other waterborne opportunists and pathogens to infectious complications of dental procedures as discussed in previous chapters. Ely *et al* [1993] has raised the question of how frequently unrecognized instances of infection might follow invasive dental surgery, where this feature of the clinical history was not elicited. Whatever the true extent of the problem, the steady accumulation of evidence of widespread and serious contamination of dental water can no longer be ignored in the context of responsible infection control in the dental office.

Chapter 4

Endotoxin contamination of dental water

INTRODUCTION

Endotoxin, a heat-stable toxin associated with gram negative bacterial cells, is composed of lipopolysaccharides (LPS) derived from the bacterial cell wall. Most of the pathogenic effects seen in gram-negative bacterial infections are mediated by endotoxin [Berczi *et al* 1993, Natanson *et al* 1994]; the associated clinical syndrome may even occur in the absence of bacteremia [Danner *et al* 1991, Graham *et al* 1994]. Endotoxin has been implicated in the pathogenesis of hepatotoxicity, hepatorenal failure, hepatic encephalopathy [Lang *et al* 1993, Odeh *et al* 1994, Shibayama *et al* 1992], periodontitis [Trope *et al* 1995, Yoshinuma *et al* 1994], mastitis [Tyler *et al* 1994], adult respiratory distress syndrome [Graham *et al* 1994, Herbert *et al* 1992, Horgan *et al* 1993], disseminated intravascular coagulation [Graham *et al* 1994], humidifier fever [Flaherty *et al* 1984, Mamolen *et al* 1993], and sick building syndrome [Teeuw *et al* 1994].

Dental unit waterlines are lined by a thriving biofilm which is composed predominantly of gram negative heterotrophic bacteria [Mayo *et al* 1990, Oppenheim *et al* 1987, Pankhurst *et al* 1990, Pankhurst *et al* 1993, Williams *et al* 1993, Williams *et al* 1994]. These organisms are released in high numbers into the dental unit coolant and irrigant water and delivered through the distal outlet of the dental instrument. With them is delivered the potential for endotoxin exposure.

In medicine, endotoxin concentrations in fluids have to be carefully controlled and United States Pharmacopoeia (U.S.P.) standards for irrigation and parenteral fluids must be observed [U.S. Food and Drug Administration 1987]. Despite the fact that there are numerous reports of gram-negative bacteria in dental water, there are no published reports on endotoxin, though there is one abstract of a presentation on the topic [Bourassa *et al* 1995]. The Centers for Disease Control and Prevention currently recommends that U.S.P. sterile water be used for all dental surgical procedures, and this stipulation requires use of "pyrogen-free" irrigant [Centers for Disease Control and Prevention 1993, United States Food and Drug Administration 1987].

Three questions were asked in this study: (1) Are there detectable levels of endotoxin in dental water, (2) Is there any correlation between the number of bacteria and the level of endotoxin present in dental water, and (3) Is endotoxin aerosolized in detectable levels by routine dental procedures?

MATERIALS AND METHODS

Water and aerosol samples

Water samples were collected from the dental lines of institutions #6 described in the previous chapter and handled and processed according to standard practices for water quality evaluation using heterotrophic bacterial plate counts [American Public Health Association 1985] as described in Chapter 1. Samples (2 to 3 mL) were collected in sterile, pyrogen-free 12 X 75 polystyrene tubes, avoiding any contact between the instrument parts and the tube during collection. Samples were shipped overnight to the laboratory with a cold pack.

Aerosols were collected using an Anderson sampling vacuum pump (Anderson Co., Atlanta, GA) and AGI-30 glass impingers (Ace Glass Co., Vineland, NJ) containing sterile water, as

described by Trudeau *et al* [1994]. In this procedure, a calibrated vacuum generator draws a measured volume of air containing the aerosol into glass vessels (impingers) set up a specific distance from the aerosol source. Over the course of a known time interval, aerosolized particles in the sample strike the surface of the sterile collection fluid within the impinger and are retained therein. In this study, impingers were set up at a distance of 60 cm from the area of dental work in a patient's mouth and a total of 0.33 cubic meters of air were sampled for each of eight tested operatories: four from operatories using ultrasonic scaler lines and four from high speed handpiece lines. Control collections done in the early morning prior to the generation of any dental aerosols were collected for comparison. Samples were shipped on ice via overnight courier and the aerosol particles retained in the collection fluid were analyzed for endotoxin.

In view of the natural day-to-day fluctuations of bacterial contamination in dental water [Santiago *et al* 1994], both water and aerosol samples were obtained on multiple occasions, ultimately providing 47 separate water-aerosol pairs for our analyses.

Bacterial contamination

Heterotrophic bacterial contamination of the water samples was assessed by plating 100 uL aliquots of serial dilutions of each sample onto R2A plates and incubating as previously described [Santiago *et al* 1994, Williams *et al* 1993]. Plates with >300 or <30 colony forming units (CFU) were considered too many, or too few to count, respectively, for statistical reasons.

At each of the 8 aerosol collection sites (four from scalers, four from highspeed handpiece lines), additional collections were made using Anderson plate chambers. In this procedure, air is drawn over the surface of agar plates through steel plates which deflect the particles onto the surface of the R2A agar for subsequent incubation and colony formation. In this way, the number of bacteria may be roughly determined and the resultant colonies permit standard microbiological isolation and identification methods to be applied.

Endotoxin Assay

The presence of endotoxin in the samples was assessed using the *Limulus* amoebocyte lysate (LAL) test [U.S. Pharmacopoeia 1993], commercially available as the Pyrotell™ kit (Associates of Cape Cod, Inc.). The LAL assay is

exquisitely sensitive to endotoxin and mandates devoted adherence to the use of endotoxin-free glassware and plasticware (including tubes and pipette tips), diluent, etc. The assay was performed in accordance with the specified protocol. Briefly, water samples were serially diluted in pyrogen-free water and a 0.1 mL aliquot of each dilution was added to 0.1 mL of LAL, vortexed, and incubated at 37C for 1 hour. A positive test result consists of the formation of a gel (as the lysate coagulates in response to the endotoxin) that is stable on inversion of the tube. This corresponds to the presence of ≥0.03 endotoxin units (EU) per milliliter. The level of endotoxin in the original sample is calculated by multiplying this value by the dilution. Commercially available endotoxin standards were used as controls.

RESULTS

Consistent with all studies to date, the extent of bacterial contamination in the dental waters sampled for this investigation far surpassed the levels associated with potable water, with counts in excess of 2.0×10^6 CFU/mL in some samples. Correspondingly, high concentrations of endotoxin (up to 15,000 EU/mL) were present.

The relationship between bacterial concentrations and the results of the endotoxin assay are shown in Figure 1. A positive correlation of 0.33 between EU/mL and CFU/mL is demonstrated, which is significant at $p < 0.05$.

Collection of aerosols from the institutional clinic dental lines resulted in the accumulation of a varied mixture of bacterial organisms which were rapidly overgrown by abundant growth of a number of fungal varieties. This precluded quantification of bacterial numbers in aerosols. Nevertheless, a variety of bacterial forms were isolated, including Gram positive cocci, Gram positive coccobacilli, Gram negative cocci, and Gram negative bacilli. Not all of the isolates were identifiable using standard microbiological and biochemical profiling techniques, but of those that could be definitively speciated, the following were found: *Staphylococcus epidermidis, Staphylococcus aureus, Staphylococcus haemolytica, Micrococcus* spp., *Micrococcus varians roseus, Pseudomonas vesicularis, Sphingomonas paucimobilis, Acinetobacter* spp., and *Flavomonas* spp. Other distinct organisms in the gram-negative pigmented colony forming categories were plentiful but did not provide satisfactory profiles in any system for identification.

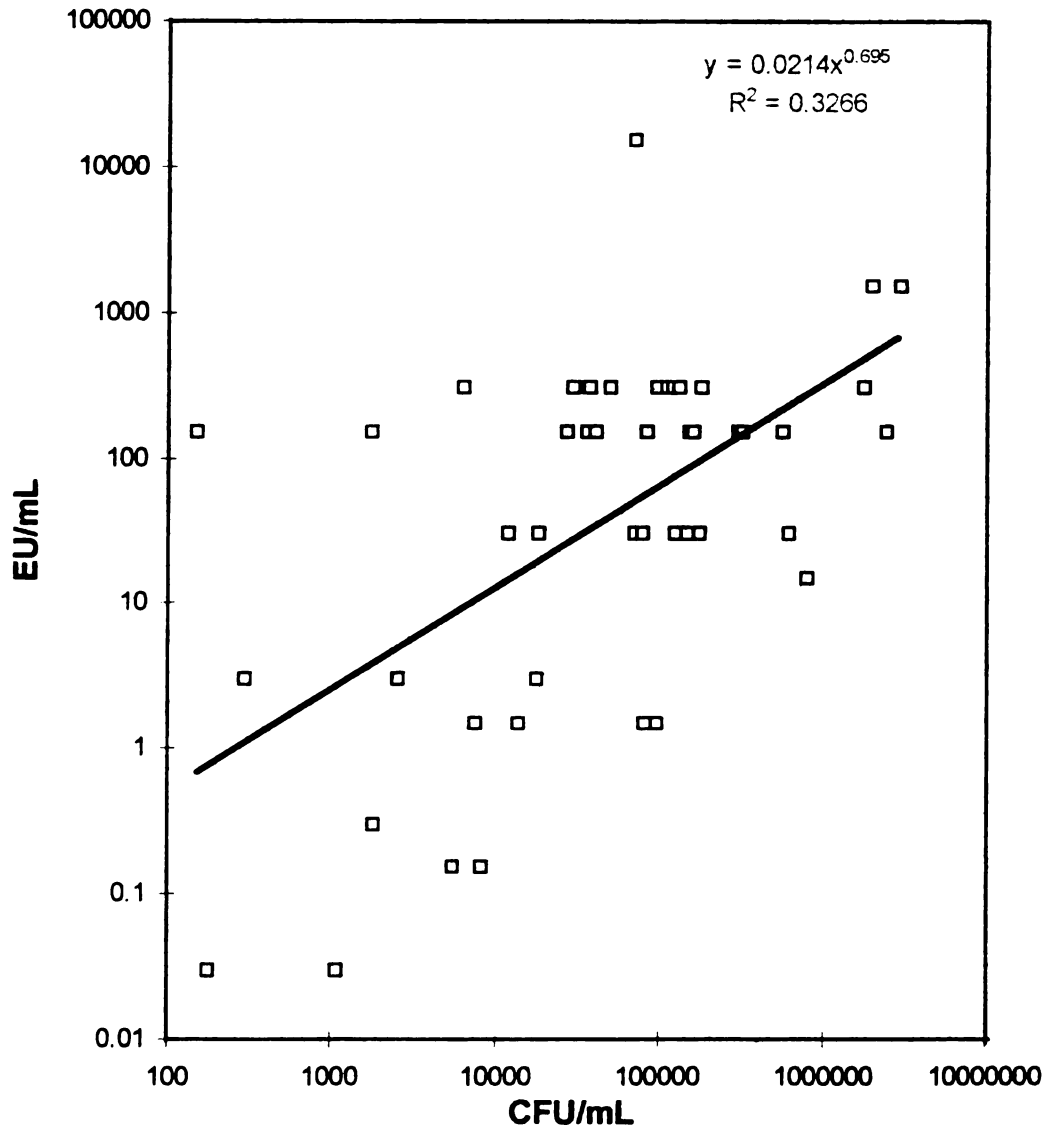


Figure 1. Correlation between bacterial contamination and endotoxin levels in dental waterline samples. A positive correlation of 0.33 between bacterial load and endotoxin level, significant at $p<0.05$, is evident.

All of the aerosol samples collected in impingers contained detectable endotoxin, though the concentration did not exceed 2.7 EU per cubic meter in any instance. No endotoxin was detectable in control air samples.

DISCUSSION

Water obtained from properly functioning distillation, reverse osmosis, and ultrafiltration systems generally has undetectable levels of endotoxin [Associates of Cape Cod 1990]. Clearly, the concentrations seen in the dental water samples far exceed any acceptable standard for water intended for medical purposes. The usually acknowledged range of 0.06 EU/mL to 0.5 EU/mL for medical devices (including liquid devices), depending on application, applies to irrigation fluids and is regulated by the federal government [U.S. Food and Drug Administration 1987].

The presence of endotoxin in dental water has potential clinical significance, both medically and dentally. Endotoxin stimulates the production of numerous cytokines which result in tissue injury [Graham *et al* 1994]. These may

inhibit healing following dental or periodontal treatment. The significance of endotoxin in the pathogenesis of periodontitis is well documented [Trope *et al* 1995, Yoshinuma *et al* 1994], and irrigation of highly vascular mucosal lesions with endotoxin-laden water during treatment for this condition is, at the very least, inappropriate.

Irrigation of the site of any dental intervention with endotoxin-laden water has the potential for introduction of the endotoxin into the patient's bloodstream. Experimental injection of endotoxin into the systemic circulation of healthy volunteers elicits the signs and symptoms of endotoxemia, including fever, elevated white blood cell count, elevated blood concentrations of stress hormones, and decreased blood oxygenation [Herbert *et al* 1992, Suffredini *et al* 1989, vanDeventer *et al* 1990].

Endotoxemia is typically associated with gram-negative infection and sepsis, but significant exposure via inhalation [Castellan *et al* 1987, Teeuw *et al* 1994] has recently been reported. Little is known about the significance of aspiration, ingestion, mucosal or dermal exposure.

Gram-negative organisms in the biofilms of plumbing and climate-control ductwork may contribute medically significant quantities of endotoxin to their surroundings [Costerton *et al* 1987, Hugenholtz *et al* 1992, Teeuw *et al* 1994]. Inhaled endotoxin significantly lowers spirometric values in otherwise healthy subjects [Castellan *et al* 1987]. Breathing endotoxin-tainted air has serious implications for exacerbation of chronic obstructive pulmonary disease and asthma in individuals with pre-existing conditions [Michel *et al* 1991]. The clinical significance in healthy individuals is not known. However, recent studies linking airborne endotoxin to "sick building syndrome" [Teeuw *et al* 1994] suggest that even uncompromised individuals are at risk for the development of medical conditions due to inhaled endotoxin. While there is no specific concentration of airborne endotoxin above which is defined as hazardous, experimental animals show clear respiratory dysfunction at 0.3 ug/m^3.

In the above study correlating airborne endotoxin to "sick building syndrome", 19 mechanically ventilated buildings were divided into "sick" and "healthy" groups, based on symptom prevalence (>15% or <15%, respectively). Airborne endotoxin levels were 6 to 7 times higher in sick buildings than in healthy ones (254 vs 46 ng/m^3), both of which were

higher than naturally ventilated buildings (35 ng/m^3). These findings suggested a significant contribution of airborne endotoxin to the etiology of sick building syndrome [Teeuw *et al* 1994]. Biofilm found in air conditioning systems may represent the source of this endotoxin [Hugenholtz *et al* 1992].

That finding is important to the work reported here: dental waterlines have lush biofilms within them [Mayo *et al* 1990], and tremendous aerosols are generated during the course of routine dental procedures [Abel *et al* 1971, Belting *et al* 1964, Earnest *et al* 1991, Hausler *et al* 1964, Kazantzis *et al* 1961, Larato *et al* 1966, Madden *et al* 1993, Stevens *et al* 1963]. The endotoxin-laden aerosolized dental water could be contributing to the development or exacerbation of numerous medical conditions, especially in occupationally exposed staff. In this study, I have demonstrated that operatory air contains detectable levels of endotoxin in addition to a variety of airborne microorganisms. Further study will be needed to determine whether these aerosols represents contamination from the waterlines, the airlines [Ely *et al* 1993], or a combination.

In light of the significant correlation demonstrated in Figure 1, the proximate source of the dental water endotoxin

contamination is likely the gram-negative organisms resident in the dental waterline biofilms [Mayo *et al* 1990, Oppenheim *et al* 1987, Pankhurst *et al* 1990, Pankhurst *et al* 1993, Williams *et al* 1993, H.N. Williams *et al* 1994]. The correlation between bacterial and endotoxin contamination levels observed in this study is at odds with recently reported findings from another laboratory [Bourassa *et al* 1995]. In that study, a correlation between the levels was sought as a "simple test for monitoring . bacterial contamination". They found no significant relationship existed between CFU and endotoxin concentrations even when CFU's varied by up to three orders of magnitude. They proposed that this lack of relationship may be due to bacterially produced inhibitors of the *Limulus* chromogenic test employed in their investigation. The LAL assay may, in fact, be less prone than the chromogenic test to such inhibitors. Other explanations could relate to sample size, variation in aliquot handling protocol and technique, or contamination of diluents or labware by exogenous endotoxins. Whatever the cause of the differences, while it is not an adequate basis on which to compute bacteria per milliliter, the correlation between endotoxin and CFU/mL is clear in this study.

In addition to the clinical implications of dental water endotoxin, there are legal ramifications. On top of the obvious malpractice liability incurred by exposing patients to massive amounts of endotoxin, there is legal risk from an employee occupational health perspective. The Indoor Clean Air Act Ammendments of 1990 mandate that within buildings, exposure to airborne pollutants cannot exceed levels of exposure in the local outside air [Hodson *et al* 1994]: dental office airborne endotoxin exposure may cross that threshold. Blaming an exacerbation of chronic obstructive pulmonary disease or asthma on workplace exposures could lead to both civil and criminal penalties.

The endotoxin literature contains an interesting report on the inhibition of *Legionella* growth within endotoxin-treated macrophages [Egawa *et al* 1992]. In this study, macrophages from a murine strain permissive for *Legionella* growth became highly resistant to growth of the organism when pretreated with endotoxin. This enhanced cytolyic activity occurs at some point subsequent to the initial bacteria-macrophage interaction. It is tempting to speculate that the high levels of endotoxin delivered in dental water might exert some sort of protective effect, contributing to the infrequency of severe legionellosis in those exposed [Fotos *et al* 1985, Reinthaler *et al* 1988, Luck *et al* 1993] to the

high levels of *Legionella* present in dental water [Atlas *et al* 1995].

In conclusion, this study has demonstrated that dental unit water contains high concentrations of endotoxin and that there is a statistically significant positive correlation between endotoxin and the bacterial load present. This water is readily aerosolized during routine dental work. Exposure to either the endotoxin-laden water or the aerosolized endotoxin represents a potential health threat. Further epidemiological and clinical studies of the consequences of dental endotoxin exposure as well as evaluation of means by which the exposure may be prevented are warranted.

APPENDIX

APPENDIX, PART 1:

Bombesin-like Neuropeptides in Nematodes: Literature Review

Filarial parasites are responsible for debilitating diseases that affect several hundred million people world-wide. Although a variety of treatments can be used to decrease the severity of symptoms, there is no effective, safe means of curing patients of filarial infection.

Not enough is known about the parasites' physiology to predict which systems or pathways may provide suitable targets for pharmacological intervention. This study was undertaken to begin characterization of a previously undescribed physiological system within the adult nematode: bombesin-like peptides and their binding sites. This system is a potential target for therapeutic interventions. By way of background, a brief review has been assembled of the major features of the filariae, the ways these confound current therapeutic approaches, and the anatomical and

physiological peculiarities that may be exploitable as targets for therapeutic interventions.

REVIEW OF FILARIAL DISEASE

At least eight species of filarial worm infect a total of over half a billion people in the world today [Wharton *et al* 1986]. The manifestations of these diseases vary widely, from the subcutaneous "Calabar" swellings caused by *Loa loa* to the grotesque elephantiasis (Figure 1) induced by *Brugia* and *Wuchereria*. These infections exact a high toll in human suffering and lost productivity from those who can least afford it: the masses living in tropical developing nations.

The life cycle of these parasites (Figure 2) is simple to comprehend, but difficult to interrupt. Microfilariae (L1 larvae) are taken up in blood meals by a biting vector (several species of mosquito for lymphatic filariae, black flies for *Onchocerca* and deerflies for *Loa*). In the vectors, they undergo two moults to the infective L3 stage which is transmitted to another human host during the next meal. The adult worms develop in the tissues, breed, and produce microfilariae. These microfilariae then migrate through the skin (in the case of *Onchocerca*) or blood

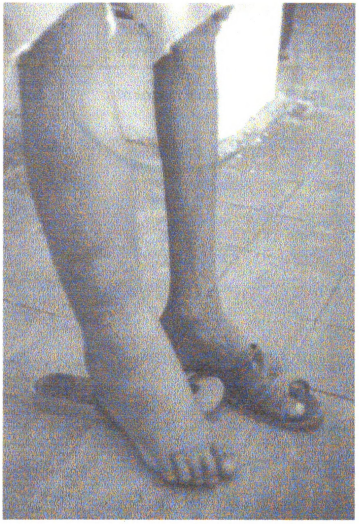


Figure 1. Sudanese elephantiasis patient (photo by MKH).

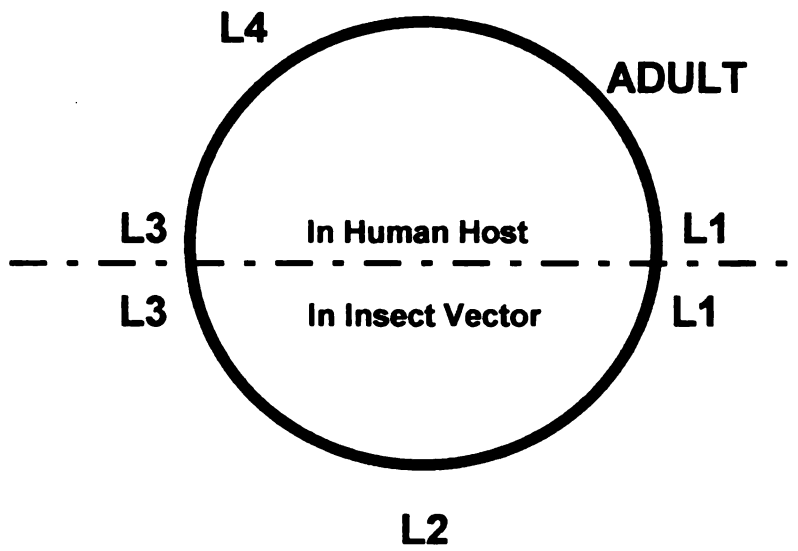


Figure 2. Filarial life cycle

(lymphatic filariae) and are eventually taken up by the insect vector.

Onchocerca volvulus causes perhaps the most dramatic filarial disease. Following transmission by the bite of an infected blackfly, the worm develops to the adult stage over the course of approximately one year. A granulomatous reaction consisting of mononuclear cells and eosinophils forms the characteristic onchocercal nodule around the adult females. Within these nodules, the parasite may live for decades [Plaisier *et al* 1991, Remme *et al* 1990]. The male worms appear to migrate between nodules, fertilizing the

females dwelling therein [Collins *et al* 1982, Duke *et al* 1990]. The gravid female produces large numbers of microfilariae which then migrate through the skin and subcutaneous tissues.

During the 6 months or so that these microfilariae remain alive, a variably effective immune response is directed against them. This appears to be due to active immunosuppression of local reactivity by the microfilariae [Akuffo *et al* 1996, Elkhalifa *et al* 1991, Soboseay *et al* 1994, Williams *et al* 1986]. However, upon their death a localized inflammatory reaction occurs. Although directed against the "foreign body" of the worm, the eosinophils, leukocytes, and interleukins called into action also cause damage to the host tissue. This small reaction, multiplied by the huge number of dead microfilariae per square centimeter of skin, can result in a very severe rash known as onchodermatitis (Figure 3).

Just as inflammation results in the skin rash, reaction to microfilariae dying in the cornea leads to punctate keratitis and scar formation terminating in sclerosing keratitis and permanent blindness at an early age (Figure 3). This expression of disease is known as River Blindness,

because infection occurs only along fast moving rivers in which the insect vector can reproduce.

In spite of the morbidity induced by *O. volvulus*, there are no safe, effective means of eliminating the infection. As is the case with most helminthic infections, the immediate goal of intervention is reducing the clinical disease, whether or not this includes elimination of the parasite. This is a legitimate and realistic goal in one sense, for symptoms in helminthiases are often proportional to the number of worms present: reduction of the worm burden can eliminate the disease state.

There is "no direct evidence of protective immunity in man against reinfection with filaria." [UNDP 1989]. Thus, despite the advent of molecular biology and the ability to clone and mass-produce select antigens, there is no prospect of an effective vaccine on the horizon. Therefore, the most promising approach to intervention is pharmacological. However, anthelmintics that are effective on luminal-dwelling nematodes are generally not effective against filariae. Often, their mode of action targets the worms' motility apparatus, resulting in paralysis and expulsion from the intestine by the host's peristalsis. Such a strategy is not effective in the tissue-dwelling worms. For

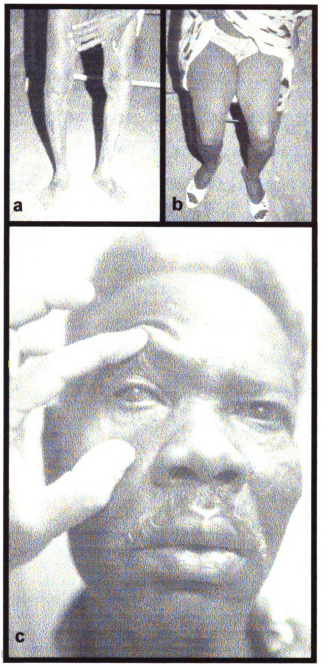


Figure 3. Sudanese onchocerciasis patients demonstrating (a) depigmentation onchodermatitis, (b) *Sowda* onchodermatitis -- note the increased pigmentation of the right leg, and (c) river blindness (photos by MKH).

this reason, other targets have been exploited, with varying degrees of success.

As can be seen in Table 1, both microfilariae and adults (macrofilariae) have been the focus of drug actions. Microfilariae are the stage most often hit by medications, and such an approach to therapy has the potential of reducing symptoms of infection as well as transmission of the parasite. Their side effects result mainly from the host's reactions to the sudden death of the larvae. This acceleration of the disease process is known as the "Mazzotti" reaction. Unfortunately, agents which exert no effect on the adult worm leave the patient open to problems caused by the next batch of microfilariae shed from the intact adults. As a result, even the best microfilaricide in use today (ivermectin) must be administered twice annually for decades to prevent overt disease. Such a requirement presents logistical problems that, in many areas where the disease is endemic, are insurmountable.

Macrofilaricides have greater potential for affecting worm populations. By eliminating the adult worm, production of microfilariae is halted. Used in conjunction with a microfilaricide to clear the patient of existing microfilariae, mass treatments with an adulticidal drug

Table 1. Overview of filaricidal agents (adapted from Subrahamanyam 1987)

Agent	Site of Action	Course of Treatment	Toxicity	Activities
Diethylcarbamazine (DEC)	Uncertain, Neuromuscular system, cuticular surface, carbohydrate and folate metabolism, host immune factors	12 days	Moderate	Microfilaricidal
Ivermectin	Uncertain, Neuromuscular system, host immune factors	1 dose	Mild	Microfilaricidal
Suramin	Carbohydrate and folate metabolism, protein kinases, intestinal epithelium	6 weeks	Severe	Macrofilaricidal
Benzimidazoles	Microtubules	3 weeks	Moderate	Microfilaricidal
Isothiocyanates & derivatives	Cuticular surface, carbohydrate metabolism, cyclic AMP phosphodiesterase, 5'-nucleotidase, aminoacyl-tRNA ligase	2-4 days	Moderate	Both
Levamisole	Neuromuscular system, carbohydrate metabolism, host immune factors	1 dose	Moderate	Both
Arsenicals	Carbohydrate metabolism, intestinal epithelium, glutathione metabolism	1 dose	Severe	Macrofilaricidal
Antimonials	Carbohydrate metabolism	1 dose	Severe	Macrofilaricidal

could eliminate the parasite from a given region in one fell swoop. Although immigration of infected humans and vectors could reintroduce infection, such an approach would be logistically more feasible than any of the other options previously discussed. Unfortunately, current macrofilaricidal agents are extremely toxic. This precludes mass treatments. Still, the idea of such an approach is appealing: what is needed is a better adulticidal drug. To get there we need exploration of filarial physiological peculiarities, and the function of the hypodermis is a good starting point.

REVIEW OF HYPODERMAL PHYSIOLOGY

Filariae, as do all nematodes, have a remarkable structure within their body wall known as the hypodermis. This subcuticular syncytium participates in a number of physiological processes, encompasses the nerve cords and excretory ducts, and is in contact with the body wall musculature. In many ways, this pluripotent structure could be considered the "brains" of the worm. Developing filariae undergo four moults. During each of these, the cuticle is at least partially replaced. The regulation of this process, which was one of the subjects of a recent comprehensive review of nematode surfaces [Geary *et al*

1995], appears to be largely under the control of the hypodermis. Some of the enzymes necessary for cuticle protein synthesis are present in the hypodermis. Other enzymes, required for the detachment of the previous cuticle from the hypodermis prior to the new cuticle's formation, are also hypodermal in origin. During the growth between moults, the proteins needed for cuticular growth again form in the hypodermis and migrate out to their proper location, though the exact mechanism of this translocation is uncertain. What triggers moulting is also unclear.

All nematodes maintain an internal turgor pressure, and are capable of osmoregulation when placed in hypotonic or hypertonic solutions. The site at which this water balance is maintained is primarily the body wall, likely the hypodermis, with only minor contributions from the gut of the worm. The mechanism of osmoregulation requires an intact metabolism to function, and it has been suggested that it may be hormonally controlled [Fuse *et al* 1993].

The cuticle is permeable to both organic and inorganic ions, with the relative permeabilities of: $K^+ > Na^+ = Cl^- >$ acetate$^-$ > gluconate$^-$ in *Ascaris suum*. The underlying hypodermis has a membrane potential of -74.9mV (E_o)/-47.6mV (E_i) which can be depolarized by increasing the [K^+] or decreasing the

[acetate$^-$] on the muscle side but not by such ionic changes on the cuticular side [Pax *et al* 1995]. This indicates that the hypodermal faces are differentially permeable to ions. The hypodermal electrochemical gradient may be the result of active ion transport and/or diffusion of metabolic organic ion by-products. Indeed, it has been shown that both occur.

The hypodermis can directly transport Cl^-. In nematode muscle, it is this ion that is principally involved in establishing and maintaining the nematode myocyte membrane potential [Geary *et al* 1995]. In contrast, mammalian or cestode myocytes maintain potentials that are dependent on potassium flux. Thus, it appears that the hypodermis is involved in regulation of the membrane potential of the body wall musculature and nerves through the control of the flow of key ions.

Filarial parasites obtain their energy from a number of different substrates via homolactate fermentation glycolysis, malate dismutation, and to some degree an electron transport system [Bryant *et al* 1989]. Intermediary metabolism of these compounds produces lactate, succinate, acetate, and formate [MacKenzie *et al* 1989]. These metabolically produced organic ions diffuse into the cuticle from the hypodermis, setting up a pH gradient [Sims *et al*

1992]. The gradient could influence the ionization states of molecules within the cuticle/hypodermis microenvironment, affecting the permeability of the body wall.

The means by which filariae take up nutrients from their environment is controversial. Although they have a functional gut, evidence is mounting that they are more dependent on transcuticular nutrient uptake rather than oral feeding [Bryant *et al* 1989, Howells *et al* 1980, Howells *et al* 1981, Howells *et al* 1983]. Similarly, filariae have a less well developed excretory system than do the intestinal-dwelling worms and it has been suggested that the cuticle/hypodermis of the former is more permeable than that of the latter to compensate for this fact [Howells *et al* 1981]. Thus, both nutrient absorption and waste excretion occur via the transcuticular route, mediated by the hypodermis [Ho *et al* 1992]. Naturally, there are limitations to transcuticular feeding and excretion. Molecules over 3000 Da in size do not traverse the cuticle [Thompson *et al* 1993]. Additionally, while the above data clearly demonstrate transcuticular absorption and excretion by filariae, there is little evidence to suggest that the orthodox view of the intestinal nematode cuticle's impermeablity to most small polar molecules is in need of revision [Masood *et al* 1983].

The hypodermis seems to be a key site in the tissue-dwelling nematode. It is involved in nutrient uptake and utilization, developmental moulting, osmoregulation, and maintenance of the internal ionic milieu. Indeed, it would seem to be the main control center of the *corpus filariae*. How these myriad of functions of the tissue are regulated is unknown. Suggestions include neuronal, hormonal, or neuroendocrine systems.

REGULATORY SYSTEMS OF NEMATODES

The nervous system of nematodes, while anatomically simple, is chemically very complex. Containing relatively few neuronal cell bodies, the basic "wiring diagram" appears to be conserved between all nematodes thus far examined, both free-living and parasitic [Geary *et al* 1992]. Unfortunately, very little is yet known about the functioning of the helminthic nervous system.

The nematode nervous system differs significantly from that of their vertebrate hosts. Anatomically, the difference is profound: nematode muscles send out processes which communicate with the nerve cord; in vertebrates, it is the nerve that sends out the axon. In contrast to vertebrate

nervous transmission, nematodes exhibit a graded rather than all-or-nothing quantal response to stimulation. The action potential so well characterized in vertebrates is absent in the worm. Nematode nerve membrane potential is primarily dependent on Cl^- flux, with the principle anions involved being Ca^{++} rather than Na^+ and K^+ [Stretton *et al* 1992, Geary *et al* 1992].

A number of neuromodulators have been found in the nervous system of nematodes. There is evidence of acetylcholinergic, GABAergic, serotoninergic, glutamatergic, and peptidergic components [Geary *et al* 1992].

If little is known about nematode neurobiology, even less is known about their endocrinology. Moulting is presumed to be controlled by three biochemically distinct categories of hormone. These are the neuropeptides which stimulate secretion of ecdysteroids, juvenile hormones which have a role in maintenance of the larval form during development, and the ecdysteroids which control the moulting process itself [Howells *et al* 1987]. Hormones are also involved in neuromuscular modulation, nutrient absorption, and osmoregulation as well [Fuse *et al* 1993, Stretton *et al* 1992].

An area of contemporary research in helminthology is peptidergic regulatory systems. Nematode nerve membrane potentials exhibit rather regular oscillations which occur in bouts, with muscle contractions occurring during the interbout intervals. This suggested the possibility that the nervous activity stimulated the release of some hormonal factor. Indeed, evidence for the presence of a large number of neurohormonal peptides in nematodes has been found [Brownlee *et al* 1993, Stretton *et al* 1992]. While helminth peptides research is a new field, this ground has proven fertile and produced some very interesting findings. One such finding was by our laboratory, demonstrating that antibombesin antiserum reacted very strongly with material at the hypodermocuticular junction zone of a filarial worm [Huntington *et al* 1993]. This discovery provided the springboard for the studies presented in this appendix.

REVIEW OF BOMBESIN-LIKE PEPTIDES

In 1970, Nakajima and co-workers isolated a peptide isolated from frogs of the genus *Rana* which they named ranatensin due to its actions on blood pressure [Nakajima *et al* 1970]. Later that year, Vittorio Erspamer and colleagues isolated a

similar peptide, bombesin, from the skin of the European frog, *Bombina bombina*. Bombesin had a number of pharmacological actions, including "hypertensive action in the dog; stimulation of the rat uterus, rat and guinea-pig colon, and the cat ileum; stimulation of gastric secretion in chicken and dog; hyperglycemic action in the rat and dog; increased insulin levels in the dog; and stimulant action on the active transport of Cl^- ions from the serosal to the mucosal side of the isolated gastric mucosa of amphibians" [Anastiasi *et al* 1970].

Other bombesin-like peptides have since been identified, including gastrin releasing peptides (GRP), neuromedins (NM), litorins, and phyllolitorins. Bombesin-like peptides have a wide distribution across the animal kingdom, from helminths [Gustafsson *et al* 1985, Gustafsson *et al* 1986, Halton *et al* 1990, Huntington *et al* 1993] and insects [Penzlin *et al* 1989] to mammals [Sunday *et al* 1987]. Tissue distribution studies have shown that these peptides may be in the central and peripheral nervous systems (including sensory centers), the gut and related organs [Sunday *et al* 1987], and are developmentally expressed in the lung [Spindel *et al* 1993].

The sequences of some bombesin-like peptides are shown below.

Bombesin: pEQRLGNQWAVGHLMa

Litorin: pENWAVGHFMa

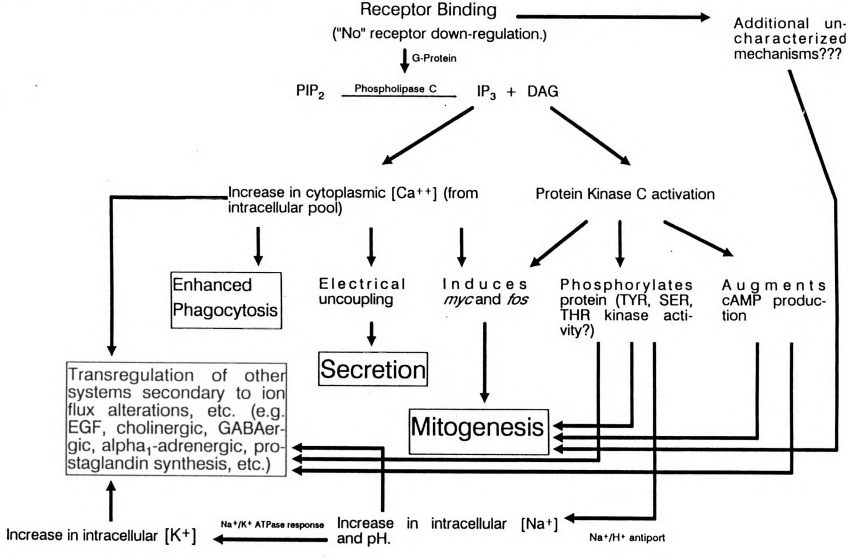
Ranatensin: pEVPNWAVGHFMa

Neuromedin B: GNLWATGHFMa

Human Gastrin-Releasing Peptide:
VPLPAGGGTVLTKMYPRGNHWAVGHLMa

As a general rule, the C-terminal 7 to 9 residues are required for specific association with the receptor [Campbell *et al* 1990]. It has further been elucidated that the C-terminal residue (methionine) is vital for initiating the biologic response but is not an essential determinant of receptor affinity [Wang *et al* 1990]. Upon binding its receptor, the bombesin-like peptide triggers a series of intracellular responses. The G-protein complex activates phospholipase C with the resulting phospholipid metabolites mobilizing calcium and activating a protein kinase. The subsequent flurry of activity is best summarized in Figure 4. Nonhydrolysible GTP analogs can block the ligand's actions, confirming the role of the G-protein. However,

Figure 7. Bombesin Cellular Physiology



phospholipid turnover is not blocked by either cholera or pertussis toxins, while mitogenesis is disrupted by them [Spindel *et al* 1993]. This suggests the possibility of additional pathways, not yet characterized, for the action of bombesin-like peptides. If analogous pathways are present in helminths, they may be susceptible to pharmacological disruption. This makes them potential targets for novel anthelminthics, particularly if they include unique components not present in their mammalian hosts.

Evidence for the presence of a member of such a potent biological regulatory family in an area of the worm having the physiological significance of the hypodermis suggested that further investigation and characterization of the system would be prudent. Hence, the following report.

APPENDIX, PART 2:

Bombesin-like Neuropeptides in Nematodes: Research Report

An area of contemporary research in helminthology is peptidergic regulatory systems. Most studies have been done on platyhelminths [Gustafsson *et al* 1985, Gustafsson *et al* 1986, Halton *et al* 1990, Halton *et al* 1994], but work on nematodes has also been revealing. Immunoreactivity to antisera against 24 different biologically active peptides has been found in *Ascaris* to date. In light of the relatively small complement of nerves present in the worm, these data "demonstrate the preponderance of the peptidergic component of the neuroendocrine system of nematodes" [Brownlee *et al* 1993]. In spite of this, to date only the FMRFamide-like peptides (FLPs) have received much attention beyond an initial immunohistological localization survey [Cowden *et al* 1993, Geary *et al* 1992, Li *et al* 1993].

Following up on the observations of bombesin-like immunoreactivity at the nematode hypodermocuticular junction zone in our laboratory [Huntington *et al* 1993], this study

was undertaken to test several hypotheses. These were (a)immunological techniques could be used to quantitate the amount of bombesin-like material within the worms, (b)binding sites/receptors specific for these molecules are present and may be localized using protocols developed by investigators studying bombesin-like peptides in other systems, (c) the kinetic properties of these binding sites/receptors may be measured using protocols developed by investigators studying bombesins in other systems, and (d)the receptor subtype may be characterized utilizing subtype-specific ligands.

MATERIAL AND METHODS

Parasite tissue acquisition

Worms were obtained from a variety of sources. Live *Ascaris suum* and *Dirofilaria immitis* were gifts from Ralph Pax and Lana Kaiser, respectively, both of Michigan State University. *Panagrellus redivivus* were grown in an oatmeal broth culture [Geary *et al* 1992]. Adult *Onchocerca volvulus* were obtained via nodule dissection and *Ascaris lumbricoides* were from freshly passed stool specimens obtained following piperazine treatment of infected humans in Zapallo Grande, Ecuador. *Haemonchus contortus* acetone extracts and *Caenorhabditis elegans* were donated

by Timothy Geary of the Upjohn Company, and *Toxocara canis* and *Toxocara cati* were donated by Robert Garrison of Purdue University. Frozen, intact *Onchocerca volvulus* and an acetone extract of *Onchcerca gutturosa* were provided by Mohamed Hag Ali and Tarig Higazi, respectively, both of the Sudan Medical Research Council. Lyophilized aliquots of API media in which *Oestertagia ostertagi* had been cultured [Douvres *et al* 1977] were provided by Bruce Hammerberg of North Carolina State University.

Radioimmunoassay

Worms for radioimmunoassay (RIA) were extracted in cold acetone for a period of several weeks, after which the extract was lyophilized. A portion of the resulting paste (20 - 200 mg) was then dissolved in RIA buffer and assayed for bombesin immunoreactivity. For this purpose, a bombesin RIA kit utilizing Tyr^4-bombesin as trace was purchased from Peninsula Laboratories^{tm}. The antisera provided with this kit was reported to have 100% cross reactivity with bombesin and Tyr^4-bombesin, tapering off to 50% for gastrin-releasing peptide (GRP). All other reagents were of the highest grade available.

The lyophilized media aliquots were not extracted, but directly dissolved in RIA buffer and assayed per kit manufacturer's instructions.

Receptor Studies

A membrane enriched preparation was made of *A. suum* and *C. elegans* using a modification of the method used by Sinnett-Smith [Sinnett-Smith *et al* 1990]. Briefly, *A. suum* were bathed in ice cold PBS containing 5 mM $MgCl_2$, 1 mM EGTA, 1 mg bacitracin/ml, 10 ug aprotinin/ml, 1 mg soybean trypsin inhibitor/ml, and 50 uM phenylmethanesulphonyl fluoride, cut into 2 cm sections, and viscera teased from the sections with forceps. The remaining tissue was then immersed in solution A containing 50 mM HEPES, 5 mM MgCl2, 1 mM EGTA, 1 mg bacitracin/ml, 10 ug aprotinin/ml, 1 mg soybean trypsin inhibitor/ml, and 50 uM phenylmethanesulphonyl fluoride, adjusted to pH 7.4, 4C, and homogenized using a glass tissue homogenizer. Intact *C. elegans* were immersed in solution A and homogenized. The homogenate was then centrifuged at 500 G for 10 minutes to remove nuclear material, cuticle fragments, and intact cells, and the supernatant was centrifuged at 30,000 G for 30 minutes. The resulting pellet was resuspended at a protein concentration of 10 mg/ml in solution A as determined by BioRad^{tm} assay, and stored in liquid nitrogen until used.

To 100 ul of binding medium composed of 50 mM HEPES, 5 mM $MgCl_2$, 1 mg bacitracin/ml, 1% BSA, adjusted to pH 7.4 was added 25-125 ug of membrane protein plus varying concentrations of ^{125}I-GRP (Peninsula Laboratories^{tm}). Following an incubation of 30 minutes at room temperature, the binding reactions were terminated by rapid filtration on an Inotech^{tm} cell harvester using glass fiber filters [1.0 um pore size] that had been presoaked in 5% polyethylenimine for 24 hours at 4C and washed with PBS containing 1% BSA immediately before use. Filters were then washed with 3 volumes of PBS containing 1% BSA at 4C and allowed to dry prior to counting on an IsoData^{tm} 105 gamma counter.

Fresh frozen whole ascarids cut into 1 cm sections, and whole filarial worms were imbedded in OCT media and frozen by immersion in liquid nitrogen. Histological sections were then prepared using a cryostat at -20C and sections were thaw-mounted on Sialylated^{tm} (*A. suum* only, for radiolabeled experiments) or poly-L-lysine coated (*A. suum, D. immitis, O. volvulus,* and *B. pahangi*, for fluorescent-labeled experiments) slides. Mounted sections were stored flat at -20C until used.

Receptor localization was initially performed using an adaptation of the methods originally used on tissues of the canine gastrointestinal tract [Vigna *et al* 1987]. Mounted sections were incubated in 10 mM HEPES (pH 7.4) at room temperature for five minutes. They were then transferred to a solution consisting of 10 mM HEPES, 130 mM NaCl, 4.7 mM KCl, 5 mM $MgCl_2$, 1 mM EGTA, 0.1% BSA, 100 ug bacitracin/ml, and 100 pM ^{125}I-GRP where they were incubated for one hour at room temperature. Slides were washed four times for 2 minutes each in 10 mM HEPES containing 0.1% BSA at 4C, then rinsed twice for 5 seconds each in water at 4C and air dried. Slides were dipped in Kodak NTB2 emulsion and exposed for 18 to 21 days in the dark at 4C until developed with Kodak D19 developer. Sections were then stained with DiffQuik^{tm} stain and examined microscopically.

Nonradioactive localization studies, using an adaptation of the method of Anton [Anton *et al* 1991], were also undertaken. Avidin-fluorescein (FITC) conjugate (Pierce^{tm}) (300 nM) was incubated with and without 100 nM biotinyl-bombesin (Peninsula Laboratories^{tm}) for five minutes at ambient temperature in subdued light. The peptide-avidin-FITC conjugate (200 uL) was pipetted onto the poly-L-lysine slides bearing cryosections of worm and incubated in the dark at 37C, 100% humidity for 30 minutes. Following

incubations, the slides were rinsed twice with 4C phosphate buffer and immediately observed on an Olympus fluorescence microscope.

RESULTS

Radioimmunoassay

Bombesin was originally isolated from a methanol extract [Anastiasi *et al* 1970]. To determine if my acetone extraction was similar to methanol extraction, I compared extracts of *A. suum* in each solvent and found no appreciable difference in extractable immunoreactivity between the two techniques (data not shown).

I found bombesin-like immunoreactivity in the extracts of free-living, luminal-dwelling, and tissue-dwelling nematodes. These include *A. lumbricoides, A. suum, B. pahangi, D. immitis, H. contortus, O. gutturosa, O. volvulus, P. redivivus, T. canis,* and *T. cati*. Quantitation of the immunoreactivity in several of these worms is displayed in Table 1. This immunoreactivity has antigenic characteristics that differ somewhat from those of bombesin. To wit, a two-fold dilution of an extract does not necessarily result in a 50% decrease in interpolated amount of peptide per assay tube. This difference is consistent

Table 2. Bombesin immunoreactivity in select nematodes.

Nematode:	pg/g extract:
Panagrellus redivivus	575
Ascaris suum	120
Ascaris lumbricoides	425
Onchocerca volvulus	460
Haemonchus contortus	625

with the idea that a peptide related, but not identical, to bombesin is present.

API media contains endogenous bombesin-like immunoreactivity (1.28 pg/mg dry weight). This is not unexpected since its complex composition includes embryo extracts which are rich in developmental mitogens, including bombesin. Culturing *O. ostertagi* in the media increases this immunoreactivity by more than 100% to well over 2.56 pg/mg dry weight, which suggests that the parasites secrete bombesin-like material into their environment.

Receptor studies

Saturable binding sites were localized to regions of the hypodermis and along the body wall muscle of *Ascaris* by

both radiolabeled and fluorescent-labeled methods (Figures 8a and 8b, respectively). Binding of labeled ligand to muscle and hypodermis is abolished in the presence of 1 uM unlabelled ligand during incubation. No evidence of specific binding is seen in the cuticle of midbody sections. Similar binding in the hypodermis of *Onchocerca*, *Brugia*, and *Dirofilaria* was also seen, while binding to the musculature of the filariae was equivocal (Figure 5c). In all organisms tested, there was non-displaceable binding of the labeled ligands to the gut.

Binding of ^{125}I-GRP to the membrane preparation of *A. suum* is saturable and specific with a K_D of approximately 3 nM and a B_{max} of 1 fmol/mg protein (Figure 6a). At 1 uM competitor, nonspecific binding is approximately 30% of total binding in the absence of competitor. Membrane preparations of *C. elegans* exhibited a K_D around 10 nM and a B_{max} of 2 fmol/mg protein with a similar level of nonspecific binding (Figure 6b). In the presence of D-Phe$_6$-Bn$_{(6-13)}$OMe, a GRP-receptor subtype specific ligand [Lin *et al* 1995, Shapira *et al* 1991], specific binding was not demonstrable.

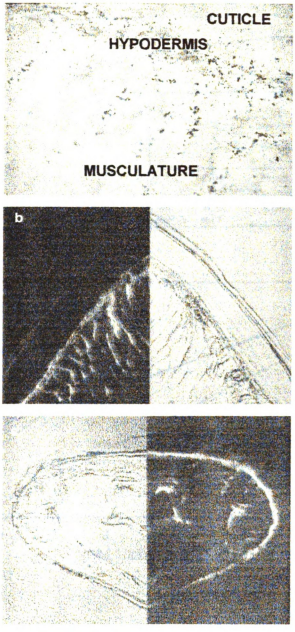


Figure 5. (a) Autoradiomicrograph of ^{125}I-GRP binding to *Ascaris* body wall. The opaque granules are the emulsion grains formed over the radioligand's binding sites. (b) Localization of FITC-bombesin binding to *Ascaris* body wall. The right shows the unstained light micrograph and the left shows the fluorescent-labeled binding sites. (c) Localization of FITC-bombesin binding to *Dirofilaria* body wall. The left shows the unstained light micrograph and the right shows the fluorescent-labeled binding sites. *Onchocerca* and *Brugia* had similar localizations.

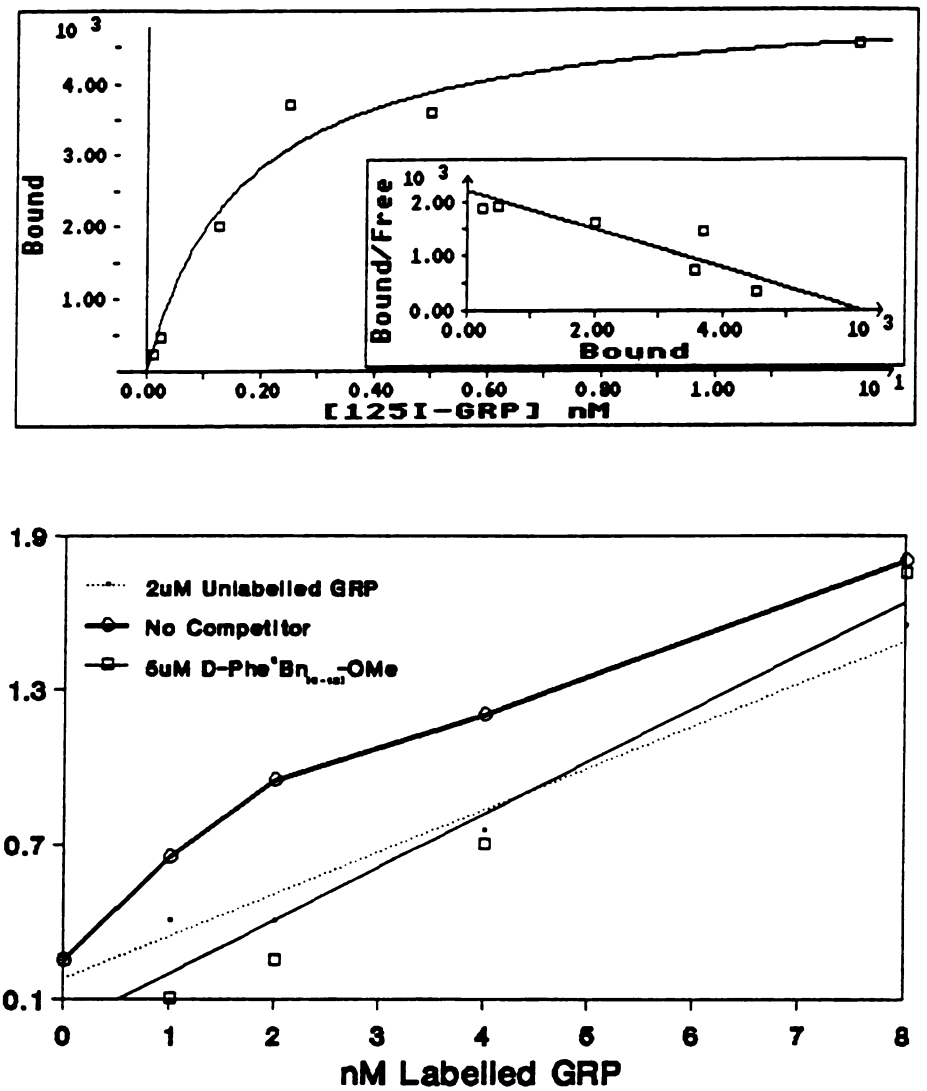


Figure 6. (a) Binding of ^{125}I-GRP to *Ascaris* membrane preparations. Inset is a Line-Weaver-Burk plot. (b) Binding of ^{125}I-GRP to *Caenorhabditis* membrane preparations. Binding in the presence of the GRP-preferring receptor subtype antagonist is not different from nonspecific binding, indicating that the site is GRP-preferring. Both the *Ascaris* and the *Caenorhabditis* preparations demonstrate saturable binding characteristics consistent with a receptor-mediated phenomenon.

DISCUSSION

Bombesin immunoreactivity was localized within the hypodermis [Huntington *et al* 1993], suggesting that the nemasin(s) (from *nema*tode bombe*sin*), may serve an important function within the region. Our RIA survey of extracts of nematodes from diverse habitats (ranging from mammalian tissues and digestive tracts to the soil) demonstrated comparable levels of immunoreactivity in all species analyzed, suggesting that whatever role the nemasin(s) serve, it is important to many, if not all, nematodes. In addition, we have found BLIR in a number of cestodes including *Taenia saginata*, *Dipylidium caninum* and *Moniezia expansa* (data not shown). This is of special interest when helminth neuropeptides are considered from a drug-discovery perspective: targeting a highly conserved system for pharmacological disruption has potential for developing broad-spectrum anthelminthic agents.

To properly characterize a peptide/receptor system, the affinity of the putative ligand for its natural receptor must be determined, and the resulting physiological responses evaluated [Burt *et al* 1985]. Pending definition of the sequence of the worm peptide(s), I have used

alternative approaches to begin a partial, but not conclusive, characterization of the nematode bombesin binding sites. That these sites are localized by both the radiolabeled GRP and FITC-labeled bombesin methods to the same areas in *Ascaris* argues against the localization being merely fortuitous. The abolition of this binding in the presence of 1 uM unlabelled peptide suggests saturable binding sites consistent with a receptor-mediated binding phenomenon. That specific binding was also displaced by D-Phe_6-$Bn_{(6-13)}$OMe suggests that these sites are of the GRP-preferring subtype. The localization to the musculature and hypodermis is, in fact, a location that might have been predicted based on knowledge of the function of those tissues, coupled with an understanding of the roll of BLIPs in other organisms. The binding sites in this study have kinetic properties comparable to those of BLIP receptors characterized from other organisms [Regoli *et al* 1991].

Clues to the function of this class of regulatory molecule in nematodes are found in the variety of physiological functions exhibited by bombesin-like peptides in other systems studied. These actions include tonotropic effects, secretagogue functions, regulation of ion flux [Anastiasi *et al* 1970], thermoregulation in mammals [Pittman *et al* 1980], immunomodulation [Amon *et al* 1993, Herdon *et al* 1993, Jin *et*

al 1990, Meloni *et al* 1992, VanTol *et al* 1993], and stimulation of mitogenesis [Cuttitia *et al* 1985, Rozengurt *et al* 1987, Szepeshazi *et al* 1992]. The localization of both the nemasins and their binding sites in the hypodermis suggests possible roles in the physiological functioning of the worm. The hypodermis is a physiologically active subcuticular syncytium that encompasses the nerve cords and excretory ducts, and is in contact with the cuticle and body wall musculature. It has been proposed that this pluripotent structure is involved in developmental moulting [Geary *et al* 1995, Howells *et al* 1980], osmoregulation [Fuse *et al* 1993], maintenance of the internal ionic milieu [Geary *et al* 1995], and nutrient uptake and utilization [Bryant *et al* 1989, Chen *et al* 1981, Ho *et al* 1992, Howells *et al* 1980, Howells *et al* 1981, Howells *et al* 1983, MacKenzie *et al* 1989, Masood *et al* 1983, Sims *et al* 1992]. Many of these functions could be mediated by the actions of bombesin-like peptides mentioned above. Additional evidence suggesting that the effects of bombesin in other systems might function similarly within the nematode systems is provided by findings that bombesin stimulates tonotropic responses in *Ascaris suum* myocytes, and crude *Panagrellus* extract elicits a statistically significant depolarization in *Xenopus* oocytes expressing the murine bombesin receptor [Thompson, personal communication].

O. ostertagia's secretion of a bombesin-like material into its environment is of potential importance in terms of the pathogenesis of parasitic infections. If the worm behaves similarly *in vivo*, the worm's peptide may act upon the host's GRP receptors, accounting for the hypergastrinemia often associated with *Oestertagia* infection. The depolarization of *Xenopus* oocytes expressing the murine bombesin receptor by *Panagrellus* extract demonstrates that activation of mammalian bombesin receptors by nemasins occurs. If other nematodes are found to secrete BLIPs into their environs, their peptides may also contribute to the pathogenesis of the variety of pathologies associated with parasitic infection.

A tremendous amount of time and effort was expended during the course of this investigation in an effort to chemically characterize the nemasins. Utilizing the RIA to monitor chromatographic fractions for the *Panagrellus* peptide, I found that it exhibits unusual behavior on reverse-phase high pressure liquid chromatography (HPLC), with no retention to the column under conditions normally used for purification of peptides (including other bombesins). As a result, other separation methods were empirically developed ultimately employing serial gel filtration/hydrophobic

interaction/gel filtration HPLC. Numerous runs (necessary due to low recoveries) of this protocol yielded enough material for an Edmann degradation sequencing attempt which was unsuccessful, suggesting a protected amino terminus. Repeating the purification quest yielded additional material which was submitted for tandem fast atom bombardment mass spectroscopy (FAB-MS-MS). This revealed a single species of molecule with a mass of 1.7 kDa, but resolution was inadequate to obtain the sequence [Huntington *et al* 1993]. At this point, the specialty column failed and was not replaced by the collaborator whose equipment we were using, aborting our sequencing efforts.

The findings communicated here, while far short of a complete characterization, clearly demonstrate the existence of a significant, previously undescribed peptidergic system in nematodes. This phenomenon is of interest from pathogenic, pharmacologic, and invertebrate physiologic perspectives and warrants further investigation.

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