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Title of Thesis: "Culture, Polymerase Chain Reaction and Restriction Fragment Length Polymorphism Studies in Bartonella bacilliformis"

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ABSTRACT

Bartonella ssp. have gained importance as etiologic agents of human disease, both in temperate and tropical regions. Reports of increasing numbers of clinical cases of bartonellosis in Peru (*B. bacilliformis*), documentation of chronic bacteremia in domestic cats with cat scratch fever (*B. henselae*), and the association of bacillary angiomatosis and parenchymal peliosis (*B. henselae* and *B. quintana*) in AIDS patients demand improved laboratory diagnostic detection and isolation techniques for this fastidious organism. We report successful culture and polymerase chain reaction (PCR) techniques applicable for this purpose. Lyophilized *B. bacilliformis* was suspended in PBS and and cultured on blood and chocolate agar plates to verify survival. Characteristic colonies were used to seed an 8% suspension of human red blood cells in RPMI 1640 media with 10% FBS and 0.7% $NaHCO_3$. Aliquots incubated at 28°C in a candle jar for 3-7 days showed numerous, pleomorphic intraerythrocytic bacteria when thin smears were stained with Giemsa. Ethidium bromide staining and visualization using ultraviolet light of fixed smears of washed red cells showed numerous fluorescent organisms within the cells. *B. henselae* was similarly cultivated and detected after incubation at 37°C. This culture system allows for early presumptive detection of *Bartonella* ssp., taking advantage of the organism's predeliction for intraerythrocytic habitation and the ability to stain fixed RBCs. For PCR, primers were designed to amplify regions between the16S and 23S rRNA genes of *Bartonella*, or the entire spacer region. In each case, the primers represented sequences conserved among *Bartonella* species, and the procedures amplified variable regions 40-1700 bp in length that should be useful for distinguishing species of *Bartonella* and for molecular epidemiology in restriction length polymorphism analysis.

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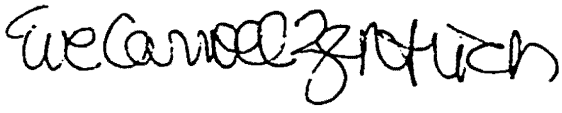

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Culture, Polymerase Chain Reaction, and Restriction Fragment Length Polymorphism Studies on *Bartonella bacilliformis*.

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INTRODUCTION

Bartonellosis, or Carrion's disease, is a bacterial infection caused by *Bartonella bacilliformis* (*B. bacilliformis*) a motile, aerobic, gram-negative pleomorphic coccobacillus. The disease, in its endemic form, occurs in remote Andean valleys of Peru, Ecuador, and Columbia between elevations of 600-3200 m and between latitude 2° north and 13° south. Bartonellosis presents in two clinical forms, as an acute febrile illness with severe hemolytic anemia (Oroya Fever) that may be life threatening, and as benign, eruptive, hemangioma-like skin lesions (Verruga Peruana). It is a vector-borne disease, with the primary vector being the sand fly *Lutzomyia verrucarum* (1). The bacteria are believed to be transmitted to humans through the proboscis of the sand fly, suggesting that the transmission is mechanical (2).

To date, the only known reservoir for bartonellosis is humans. No animal or plant reservoir has been identified. In endemic areas, it is believed that ten to fifteen percent of patients with the skin lesions (Verrugas) are chronic bacteria carriers, demonstrated by a positive blood smear. It has been estimated that approximately 60% of asymptomatic inhabitants of endemic areas have antibodies against *B. bacilliformis* (2).

Clinically, Bartonellosis has three distinct stages, that may reflect the host's immunity to *B. bacilliformis*. Any of the stages may be absent. The incubation period averages 21 days, but may be longer if the anemic stage is absent. Which of the three stages present, and their severity, vary with age (immune infant under 6 months, child, or adult) and immune status (resident or outsider) (1). Oroya Fever (the anemic stage) is caused by the bacterium penetrating into red blood cells by a process of forced endocytosis. This stimulates the mononuclear phagocytic system to take up the parasitized red cells in enormous numbers, resulting in a rapidly progressive and severe intravascular hemolytic anemia. The anemia is typically macrocytic and hypochromic, and up to 100% of circulating red blood cells may be parasitized by several bacteria. There is heavy infiltration of the reticuloendothelial system, believed to be caused by a combination of forced endocytosis and phagocytosis of the parasitized red blood cells. The infiltrated reticuloendothelial cells are characterized by the formation of large colony masses in their cytoplasm, which causes distension and partial luminal occlusion, the major pathognomonic histological change seen in the acute hemolytic stage. Other symptoms include pallor, general malaise, low-grade (septic type) fever, and enlargement of the lymph nodes, liver, and spleen. Mortality in the pre-antibiotic era

ranged from 40% to as high as 90% (1,2,3,4). At the end of the septicemic stage, variable degrees of immune deficiency are seen, probably due to transient immunosuppression resulting from reticuloendothelial system overload (2). Superinfections with opportunistic bacteria (particularly gram negative organisms such as Salmonella or Shigella), parasites, and viruses may complicate recovery (5).

The final manifestation of Bartonellosis is the eruptive, cutaneous stage, known as Verruga peruana. The Verruga are superficial and/or subcutaneous nodules, which usually appear within the first 2 months after the hematic phase. The lesions are round, highly vascular, red to purple in color, and appear in crops, particularly on the extensor surfaces of the extremities and on the trunk and face. Headaches, as well as bone and joint aches, may precede the outbreak. The cutaneous phase is self-limited, and the lesions do not leave scars unless they become superinfected. Verrugas may occur in patients, usually from endemic areas, who did not experience a clinically evident septicemic first stage. This may reflect partial immunity against the bacteria due to constant exposure and/or passive maternal immunity. *Bartonella* bacteria are found in the interstitum and within the cytoplasm of endothelium cells in the lesions and it is believed they produce a substance responsible for the distinct vascular proliferation seen in the Verruga. On histologic examination, the endothelial cells of the Verruga show cytological atypia from the terminal vasculature of the dermis and subcutaneous tissue. It is now known that the *B. bacilliformis* releases an angiogenic factor that stimulates human endothelial cells to proliferate and to release tissue plasminogen activator (1,2,3,4).

Bartonellosis was first accurately described in 1858 by the Peruvian doctor Tomás Salazar, but descriptions of the biphasic disease range back from journals kept by conquistadors to ancient artifacts (3,6,7,8). The disease existed and was depicted on vases and other ceramics from over 2000 years ago. A pre-Columbian mummy was found that demonstrated the typical morphologic features of Verruga peruana in tissues from it that had been rehydrated. Pedro Pizarro in 1571 was able to distinguish between bartonellosis and syphilis by the appearance of the lesions. Herrera y Tordesillas in 1601 described an outbreak of Verrugas that attacked the Spainiards in Peurto Viejo, Ecuador, not to mention the many other early explorers that recorded outbreaks of the disease on their arrival to South America (3,7).

The potentially deadly anemic phase of bartonellosis became known as Oroya fever from an outbreak that occurred in 1871. A high mortality epidemic occurred among laborers constructing

the railway line between Lima and La Oroya in 1871. Many of the laborers that survived the Oroya fever went on to develop Verruga peruana, suggesting to some investigators that the two disease were related. The two phases of bartonellosis were conclusively linked in 1885. Bartonellosis earned the name Carrion's disease from a Peruvian medical student, Daniel Alcides Carrion. In 1885, Carrion linked the two phases of the disease through self-experimentation. Carrion inoculated himself with blood taken from the eruption of a patient with Verruga peruana. The purpose of his self-experimentation was to study the prodromal signs and incubation period of Verruga. Three weeks into the experiment, Carrion fell ill with fever and developed progressive anemia. A few days before he died Carrion realized that he was suffering from Oroya fever. Carrion's self-experiment provided the proof that Oroya fever and Verruga peruana had the same origin (3,9). Although the clinical presentations of bartonellosis had been described and linked, it was not until 1905 that the causative agent, *B. Bacilliformis*, was characterized (6).

Recently, two additional *Bartonella* species, *Bartonella henselae* (*B. henselae*) and *Bartonella quintana* (*B. quintana*) have been recognized as causing disease in North America. Cat scratch disease (fever) was first described in 1931, but it was not until 1990 that the causative agent, B. *henselae*, was characterized. Bacillary angiomatosis was first described in 1982, but its causative agents, *B. henselae* and *B. quintana*, were not characterized until 1990 and 1991, respectively (6,10,11). Although only recently linked with bacillary angiomatosis, *B. quintana* was originally identified as the louse-borne agent of trench fever, a significant cause of morbidity among Allied troops serving in France during World War I (WWI). Trench fever was studied intensely by military researchers during and immediately after WWI. Work ceased, however, when the organism no longer was a threat to troops due to changes in fighting tactics (specifically, the move away from trench warfare). Additional *Bartonella* ssp. that have been identified include *Bartonella elizabethae* (*B. elizabethae*) and *Bartonella vinsonii* (*B. vinsonii.*). *B. elizabethae* is a suspected cause of endocarditis, while *B. vinsonii.* has not been linked to any specific disease (12,13). It is of importance to note that before 1993, *B. henselae*, *B. quintana*, *B. elizabethae* and *B. vinsonii.*were all known by the genus *Rochalimaea.* Evolutionary genetic studies based on 16S ribosomal RNA (rRNA) subunit genomic region revealed how closely related these organisms were to *Bartonella bacilliformis*, and the genera *Bartonella* and *Rochalimaea* were combined under the name *Bartonella* (6).

The advent of HIV infection has brought new manifestations of old pathogens, as well as

the recognition of new infectious agents causing previously undescribed lesions. This is the case for *B. henselae* and *B. quintana* infections, which were identified as the causative agent for unique vascular proliferative lesions, known as bacillary angiomatosis (BA). BA is an infectious disease of the skin, bone, liver, and other organs of HIV-infected people. Stoler and coworkers made the initial report of BA in 1983. They described an AIDS patient with multiple subcutaneous tumors accompanied by fever, sweats, and weight loss. Histological evaluation of the lesions revealed a new gram-negative, Warthin-Starry-staining, fastidious bacillus. Initial histopathological data suggested that causative agent of BA might be the cat scratch disease (CSD) bacillus. Warthin-Starry stain and electron microscopy revealed bacteria within the BA lesions of HIV-infected patients that appeared similar to the CSD bacillus. Relman et al. provided the first information on the genus of the organism that causes BA. Using bacterial DNA extracted from cutaneous and splenic lesions of BA and molecular techniques, they found that the organism was distinct but closely related to *B. quintana*. At the same time, Slater et al. reported several cases of bacteremia in HIV-infected patients. The organism isolated from these patients was a small gram-negative bacteria, and it was found to be the same as the identified by Relman's group. The bacterium was designated as a new species, *B. henselae*. Further identification of bacteria isolated from cutaneous BA lesions revealed that both *B. henselae* and *B. quintana* could cause cutaneous lesions, while *B. quintana* could also cause osseous lesions (12).

The manifestations of BA include diverse cutaneous lesions, visceral parenchymal bacillary peliosis of the spleen and liver, and involvement of a single or multiple organ systems. *B. henselae* and *B. quintana* have been cultured from the spleen and cutaneous lesions of BA patients, as well as the blood in patients with visceral and cutaneous BA. BA is treatable with antibiotics, erythromycin being the drug of choice. It is interesting to note that the clinical and histological features of BA and Verruga peruana are virtually identical (12,13,14).

Cat scratch disease was first described in 1931 by Robert Debre, but it was not reported in the medical literature until 1950. The etiological agent of CSD was not identified until 1988 by D.J. Wear et al., when a candidate bacterium was viewed in the lymph nodes of patients with CSD using the Warthin-Starry silver impregnation stain. In the same year, English et al., were able to grow the organism from lymph node cultures of ten patients with CSD. In 1991 the organism was named *Afipia felis* (*A. felis*) by Brenner et al., however, it became apparent that the majority of CSD patients were infected with a gram-negative organism that resembled *A. felis*, yet was not

culturable using the same methods that yielded *A. felis*. In addition, these patients did not exhibit an immune response to cultured *A. felis*. In 1992, two laboratories (Regnery et al., and Welch et al.) announced the discovery of a new etiological agent for CSD, *B. henselae*. Although it is now believed that both *A. felis* and *B. henselae* are etiological agents of CSD, the majority of cases appear to be caused by *B. henselae* (10,15).

CSD is for the majority of patients a benign, self-limiting, subacute regional lymphadenopathy that is usually preceded by an erythematous papule or pustule at the site of dermal inoculation. Low-grade fever, malaise, and generalized achiness are seen in many cases, and headache, anorexia, and spleenomegaly may also present. Up to 2% of the patients (the majority of this 2% being children under the age of 12) may experience complications involving the liver, spleen, lung, bone, or skin. Complications involving the central nervous system are considered the most severe, and present with convulsions, confusion, combative behavior, and coma, but they usually resolve in a few days, without sequlae, with supportive care. Histological findings in the involved lymph nodes are stellate caseating granulomas, microabcesses, and follicular hyperplasia. Antimicrobial treatment is generally not recommended for CSD since most typical cases do not respond to antimicrobial administration. In most cases of CSD, the disease resolves spontaneously in a few weeks to months, with supportive care given in response to the symptoms (10,16,17,18). It is interesting to note that in immunocompetent patients, *B. henselae* infection usually manifests as CSD, while in immunocomprimised patients (i.e., HIV-infected), it usually presents as BA.

The reservoir for *B. henselae*, the causative agent of BA and CSD, is believed to be the domestic cat (*Felis domesticus*). Cats have been shown to experience prolonged, asymptomatic (making it difficult to identify infected cats since they appear healthy) bacteremia, which would make them a persistent reservoir. Studies have shown a direct association between *B. henselae* infection in humans and exposure to pet cats with *B. henselae* blood infection. It is thought that *B. henselae* are introduced from infected cats into humans by way of a bite or scratch. Additional work has implicated the cat flea (*Ctenocephalides felis*), taken from infected cats, as a potential vector of *B. henselae*. Antibiotic treatment of infected cats and control of fleas are potential strategies for decreasing human exposure to *B. henselae* (16,17).

The recent recognition of *B. henselae* and *B. quintana* as the etiologic agents for cat scratch disease and/or bacillary angiomatosis, and the recent increase in the public health significance of

these two diseases, has lead to *Bartonella* species (ssp.) being designated an Emerging Disease Pathogen by the Centers for Disease Control and Prevention (19). Since *B. bacilliformis* causes a similar (but distinct) acute and chronic disease, which includes both North American clinical syndromes, it bears close study as it relates to the emergent North American disease. Most of the work available on bartonellosis is dated and consists of case reports, descriptions, and hypotheses on the disease phases, possible vector, and epidemiology. Few field epidemiologic studies have been done (most studies have dealt with hospitalized patients or hospital records), and little is known about the risk factors for developing bartonellosis.

The *Bartonella* ssp. responsible for human disease have not been positively identified until recently due to the fact that traditional methods for identifying microbial pathogens rely on cultivation or purification of the organism. Since *Bartonella* ssp. are fastidious and slow-growing, these techniques often failed. It was not until the advent of molecular techniques that allowed for analysis of certain genetic sequences that these organisms were able to be identified and placed within a phylogenetic tree. The majority of the sequence work that has been done is with the 16S ribosomal RNA (rRNA) subunit. Although all genetic sequences accumulate mutations over time, some sequences accumulate these changes in a manner that makes them useful for evolutionary studies. The most useful sequences are those that encode molecules with essential biological function. These sequences are fairly consistent, regardless of the organism they come from. The most commonly used of these types of sequences is the 16S rRNA molecule. Bacterial 16S rRNA gene sequences are ideal for these types of studies since they contain interspersed highly conserved regions as well as highly variable regions. Some of the conserved sequences are common to all previously studied bacteria, but are not found in human rRNA gene sequences, making it possible to pick these sequences out from human DNA using polymerase chain reaction (PCR) (20,21).

Current work (past ten years) on *B. bacilliformis* has dealt with studying the immunologic, molecular, and angiogenic properties of the bacteria. Unfortunately, all of this work has still to: 1) produce a rapid culture system for the fastidious organism; 2) yield a rapid immunologic/antigenic assay to detect infection; 3) confirm the vector and its role in the spread of the disease; 4) pinpoint the potential reservoir host(s); and 5) specifically define the epidemiology of bartonellosis. The molecular work that has been done with the organism, however, has opened doors. PCR and restriction fragment length polymorphism (RFLP) techniques have opened up new areas of exploration on this disease. PCR and RFLP methods allow for the rapid (compared to culture) and

accurate (compared to conformation using a stained blood smear) detection of infection with *B. bacilliformis*. RFLP assays also allow for the determination of strain variations, important for molecular epidemiology studies. The ability to subtype *B. bacilliformis* by a PCR-based RFLP method into different groups would have major implications for future epidemiological studies. PCR-based subtyping methods are particularly attractive for *Bartonella* ssp. since they are fastidious and slow-growing, and other molecular subtyping methods (such as multilocus enzyme electrophoresis, genomic DNA fingerprinting, etc.) require large numbers of cells for enzyme of DNA extraction (20,21). PCR studies could also be used to confirm the vector(s) of bartonellosis, along with the potential reservoir(s). The following work was done to develop specific PCR and RFLP techniques for use in the lab to detect and characterize *B. bacilliformis* bacteria in blood or other samples from humans, insects, and animals. It was hoped that these assays would aid current work to further define the epidemiology, vector, and potential reservoir(s) of human bartonellosis.

METHODS and MATERIALS

BLOOD CULTURES and SLIDE PREPARATION

Lyophilized *Bartonella bacilliformis*, strain KC583 (*B. bacilliformis*, KC583), and *Bartonella henselae*, strain Houston (*B. henselae*, Houston), were obtained from American Type Culture Collection (ATTC, Rockville, MD). It is presumed that the bacteria were originally isolated from cases, rather than vectors. The lyophilized bacteria were suspended in 10.0 mL of phosphate buffered saline (PBS) and cultured on 5% nutrient sheep red blood cell (blood agar plate) and nutrient chocolate agar plates (chocolate agar plate) (Remmel) by adding 1.0 mL of the suspension to the plates. The unused suspension was stored at 4°C. An additional 3.0 mL of PBS was added to each plate to prevent them from drying out during extended incubation. All cultures were incubated in candle jars, with *B. bacilliformis* at 27°C and *B. henselae* at 37°C for 7-14 days (1,2). Care was taken during this time to prevent the plates from drying out by adding additional PBS to the plates as needed. In addition, care was taken during handling of the plates to prevent contamination (due to the need for extended incubation).

An isolated colony from either the blood or chocolate agar plates was used to seed a liquid media that was modeled after the media used for culturing malaria parasites *in vitro*. The media

consisted of an 8% suspension of washed human red blood cells (hRBCs) (Valley Biomedical, type A-, or Interstate Blood Bank, type O+) in RPMI 1640 media (GibcoBRL) with 10% fetal bovine serum (FBS) (GibcoBRL) and 0.7% sodium bicarbonate ($NaHCO_3$) (Sigma Biosciences) (complete media). The media was than aliquoted in 5 mL amounts onto 60 mm^3 tissue culture plates, and incubated in a candle jar in a tissue culture incubator set at 27°C. The cultures of *B. bacilliformis* and *B. henselae* were subcultured once a week (approximately every 7 days) by adding 0.1mL of *Bartonella* ssp. infected hRBCs to a fresh 8% suspension of hRBCs in complete media. Periodically, aliquots of each culture were removed for preservation by adding dimethyl sulfone (DMSO) (Sigma Chemicals) to the aliquot to 28%, followed by immediate freezing in liquid nitrogen. Every other week, and before freezing down for preservation, the cultures were resuspended and a smear made on a nutrient agar plate (Remmel) to check for contamination. The nutrient agar plates were incubated at 37°C in a 5% carbon dioxide atmosphere for 48 hours. Since *Bartonella* ssp. require red blood cells (RBCs) for growth, any growth seen on the nutrient agar plates after 48 hours was considered contamination. The nutrient agar plates were sent to the microbiology lab for identification of the contaminate, and the contaminated *Bartonella* ssp. cultures were destroyed.

At each subculturing, blood smears were made to monitor the growth of the *Bartonella* ssp. in culture. Thin smears were air dried, fixed with methanol, and stained for 30 minutes in 10% Giemsa (Fischer Chemicals). After 7 days in culture, numerous, pleomorphic intraerythrocytic bacteria were seen. Ethidium bromide (Sigma Molecular Biology) staining was also done. The culture was resuspended and a 200 mL aliquot removed to a polypropylene microfuge tube. To the culture aliquot, 200 mL of 200 mg/mL ethidium bromide in PBS was added, and the mixture was incubated in a 37°C water bath for 45 minutes. A thin smear was pulled and air dried. The slides were stored at 4°C in the dark until viewing under a fluorescent microscope.

DNA EXTRACTION.

B. bacilliformis, KC583 and clinical samples' genomic DNA was isolated using the following basic protocol (3) for the preparation of genomic DNA from bacteria. Basically, bacteria from a saturated liquid culture are lysed and proteins removed by digestion with proteinase K (Sigma Biosciences). Cell wall debris, polysaccharides, and remaining proteins are removed by

selective precipitation with CTAB (Sigma Molecular Biology). The high molecular weight DNA is recovered from the resulting supernatent by isopropanol precipitation. The extracted genomic DNA was then digested with the restriction endonuclease *Eco* RI (New England Biolabs) to yield genomic fragments of smaller molecular weights. This DNA served as the template for PCR reactions involving *B. bacilliformis*, KC583 and clinical samples. Template DNA for *B. bacilliformis*, KC584 was obtained from Dr. Micheal Minnick (Division of Biological Sciences, The University of Montana) and consisted of a *Sph* I digested clone of the 16S-23S intergenic spacer region (906 nucleotides) in a pUC19 vector.

PRIMER DESIGN

For PCR, three sets of primers were designed to amplify different regions of the *Bartonella* ssp. 16S and 23S rRNA. Primers were designed using sequence information obtained from GeneBank of the 16S-23S intergenic spacer region of *B. bacilliformis*, KC584 (4,5). The first two sets of primers (primer set 1 and primer set 2) amplify areas within the intergenic spacer region that contain conserved areas of homology, along with highly variable regions. This allows for the primers to match up with the regions conserved among *Bartonella* ssp., while the variable regions will be used for RFLP analysis. The third set of primers (primer set 3) was designed to amplify the entire 16S-23S rRNA intergenic spacer region, with a *Hind* III site incorporated into the sense primer and an *Eco* RI site in the antisense primer to allow for cloning and sequencing of this area.

Primer set 1 consisted of a 22 base pair (bp) (bp 13-27) sense primer, and a 22 bp antisense primer (bp 419-442). Primer set 2 consisted of a 22 bp (bp 406-427) sense primer, and a 25 bp antisense primer (bp 1207-1231). Primer design and expected products (for both primer set 1 and 2) were based on sequence information obtained from GeneBank accession L26364. Primer set 3 consisted of a 26 bp sense primer (bp 1160-1186 of the 16S sequence), and a 24 bp (bp 465-489 of the 23S sequence) antisense primer. Primer design was based on sequence information from GeneBank accessions M65249 (16S rRNA gene), L26364 (intergenic spacer region), and L39095 (23S ribosomal gene) (4,5).

POLYMERASE CHAIN REACTION.

The basic PCR amplification mix for reactions involving primer set 1 or 2 contained in a total of 100 µL, 1 µmol of each primer, 200 µM of each deoxyribonucleotide, 4 mM $MgCl_2$, 10 ng of template DNA, and 1U of *taq* polymerase (Perkin-Elmer Cetus). Prior to amplification, each

sample was heated at 94°C for 5 minutes, during which time the polymerase was added. Amplification conditions for a total of 30 cycles consisted of denaturation at 94°C for 1 minute, annealing at 55°C for 1 minute, and extension at 72°C for 1 minute, except for the last extension cycle, which was 72°C for 7 minutes followed by a hold at 4°C. The amplification mix for reactions involving primer set 3 contained in a total of 100 µL, 1 µmol of each primer, 200 µM of each deoxyribonucleotide, 1.5 mM $Mg(OAc)_2$, 100 ng of template DNA, and 1U of *rTth* polymerase (Perkin-Elmer Cetus). Prior to amplification, each sample was heated at 94°C for 5 minutes, during which time the polymerase was added. Amplification conditions for a total of 35 cycles consisted of denaturation at 94°C for 2 minutes, annealing at 55°C for 1 minute, and extension at 72°C for 2 minutes, except for the last extension cycle, which was 72°C for 7 minutes followed by a hold at 4°C. Samples yielding an amplification product of the expected size on agarose gel electrophoresis were considered positive. All PCR reactions were performed in either a Perkin-Elmer GeneAmp 9600 or GeneAmp 2400, using either 0.6 or 0.2 mL GeneAmp tubes.

AGAROSE GEL ELECTROPHORESIS

Mobility of PCR amplification products and RE digests were determined by comparison with a mixture of λ RF DNA/*Hind* III fragments and ϕX174 RF DNA/*Hae* III (GibcoBRL) fragments on a 0.5 mg/mL ethidium bromide stained agarose (Sigma Molecular Biology) gel. Gels were run in TAE buffer, with the average run at 70 volts for 1 1/2 hours (2). Depending on the estimated size of the DNA to be electrophoresed, the concentration of the agarose was varied from 0.8% for high molecular weight samples, 1.0% for middle weight samples, and 1.5% for small molecular weight samples. Wells were loaded with 5 µL of the λ RF DNA/*Hind* III fragments and ϕX174 RF DNA/*Hae* III size marker or with 10 µL of sample plus 2.5 µL of 5x loading buffer. Gels were photographed and analyzed using a digital camera and the Molecular Analysis software program. Since the concentration of the DNA in the size marker was known, 500 ng of λ RF DNA/*Hind* III and 250 ng of ϕX174 RF DNA/*Hae* III per 5 µL, estimates of the concentration

of the DNA in the sample volumes could be made.

RESTRICTION ENZYME (RE) DIGESTIONS

In general, digestions with restriction endonucleases (New England Biolabs) were setup according to the manufacturer's specifications. The digestions were performed in a minimum volume of 20 μL to ensure adequate dilution of the glycerol the RE enzymes were packaged in, and to obtain adequate volume for running a minimum of two electrophoretic gels and/or a double RE digestion. The RE digests were incubated in a 37°C water bath for an average of 4 hours (incubation time could vary between 2-6 hours depending on the type of digest and DNA involved). Digests were analyzed using agarose gel electrophoresis.

PURIFICATION of DNA

Several methods of purifying DNA fragments from contaminates were used. The first method consisted of purifying DNA fragments using DE81 paper. Basically, DNA in the gel was visualized using longwave ultraviolet light. The band of interest was cut from the gel, and a piece of DE81 paper the same size as the agarose block containing the DNA of interest was cut. Several blocks were cut out of a clean agarose gel, and the DE81 paper and block of interest were sandwiched into the cut out portion of the gel. The gel was then run for 15 minutes at 180 volts, or until the DNA of interest had migrated onto the paper. The DNA was then eluted from the DE81 paper with salt washes and precipitated using ethanol. This method was found to be the most time consuming and least efficient (due to high sample loss) of the purification methods.

The most efficient method of purifying DNA consisted of using a kit containing a minicolumn and DNA-binding resin. Depending on the source and type of DNA, either Promega's Wizard PCR Preps DNA Purification System for Rapid Purification of DNA Fragments or Wizard DNA Clean-Up System was used. The protocols provided by the manufacturer were followed.

CYCLE SEQUENCING

Perkin-Elmer Corporation's ABI Prism Dye Terminator Cycle Sequencing Core Kit was used to generate sequencing data. When used in conjunction with specific template preparation techniques outlined by the manufacturer, the cycle sequencing protocol gives sequence data for single-stranded (ss) and double-stranded (ds) DNA templates and PCR fragments to approximately 400 to 650 bases, depending on the size of the sequencing gel used. The protocol was designed to work primarily with all Perkin-Elmer thermal cyclers.

Briefly, a reaction premix of kit reagents was prepared, containing 16 μL of 5x sequencing buffer, 4 μL dNTP mix, 2 μL A dye terminator, 2 μL C dye terminator, 2 μL G dye terminator, 2 μL T dye terminator, and 4 μL AmpliTaq DNA polymerase, FS, for a total of 32 μL per 4-reaction mix. The premix was stored at -20°C until use. Each cycle sequencing reaction was carried out in a final volume of 20 μL, and consisted of 8.0 μL of the reaction premix, 0.5 to 6.0 μL of template (depending on the type and concentration of the template), 3.2 pmole of primer, and distilled water (dH_2O) q.s. The cycle for sequencing on the GeneAmp 2400 consisted of a rapid thermal ramp to 96°C, 96°C for 10 seconds, rapid thermal ramp to 50°C for 5 seconds, rapid thermal ramp to 60°C, and 60°C for 4 minutes. This complete cycle series was repeated for 25 cycles. At the completion of the 25 cycles, there was a rapid thermal ramp to a 4°C hold.

Excess dye terminators were removed from the extension products by either ethanol precipitation or with a Centri-Sep spin column. The manufacturer's protocol was followed for the spin columns. For ethanol precipitation, 2.0 μL of 3 M sodium acetate, pH 4.6 and 50 μL of 95% ethanol were added to a 1.5 mL microfuge tube. The entire 20 μL reaction mix was than added. The microfuge tube was vortexed, and than placed on ice for 10 minutes. Next, the tube was centrifuged at maximum speed for 20 minutes and the ethanol solution was removed being careful not to disturb the pellet. The pellet was than carefully washed with 250 μL of 70% ethanol. The wash was removed and the pellet was vacuum dried and stored at -20°C until further use.

LIGATION

Ligation of the insert DNA of interest (amplicon 3, the PCR product of primer set 3) to plasmid Bluescript (+/-) phagemids (pBS) (GibcoBRL) was performed using T4 ligase and its buffer (GibcoBRL). The basic protocol followed for setting up the ligation reaction was that provided by the manufacturer of the pBS used. Before ligation of the insert to the plasmid, each was double RE digested with *Hind* III and *Eco* RI to generate complementary sticky ends. The digestions were cleaned using the Wizard DNA Clean-Up System (to purify the DNA of interest away from the REs and small DNA fragments generated from the digestion). In general, the ratio

of the μg concentration of insert to plasmid vector was at least 2:1, but could range as high as 10:1 depending on the quality of the DNA used. The ligation reactions were incubated for 2 hours at 25°C (room temperature), and than stored at -20°C until use. Ligations were set up similar to RE digests.

TRANSFORMATION of CELLS

MAX Efficiency DH5α Competent Cells (GibcoBRL) were thawed from -70°C on wet ice. At the same time, the required number of polypropylene tubes were placed on ice to chill, and DNA from the ligation reactions was diluted in TE buffer to give a final concentration of 1-10 ng of DNA. Once thawed, the cells were mixed by gently tapping the sides of the tube, and a 50 μL aliquot of cells was added to the chilled polypropylene tubes along with 1-5 μL of the diluted ligation mix. A control transformation consisting of pBS that had been ligated back onto itself was set-up to check transformation efficiency. The cells plus the ligation mix were than incubated on ice for 30 minutes, followed by a 45 second heat shock in a 42°C water bath. The cells were than placed on ice for 2 minutes, 450 μL of room temperature S.O.C. medium (GibcoBRL) was added, and the tubes containing the transformation mix were placed in a shaker incubator set at 225 rpm and 37°C for 1 hour. During the hour incubation, the plates for the tranformants were prepared. LB agar plates containing 100 μg/mL of ampicillin were warmed to room temperature. Each plate had 100 μL of 40 mM isopropyl-β-D-thio-galactopyranoside (IPTG) and 100 μL of 2% 5-bromo-4-chloro-3-indoyl-β-D-galactopyranoside (X-gal) spread evenly on them by shaking 5-7 sterile glass beads over the surface of the plate to spread the substrates. The transformation mixes were diluted as necessary and 100 μL of the dilution spread on the plates using the glass beads. Excess transformation mix was stored at 4°C, and could be used up to 4 days after it had been prepared. Plates were incubated overnight (O/N) (usually no more than 17 hours) at 37°C.

MINI- and MIDIPREPS of PLASMID DNA

Miniprep cultures were setup by inoculating 3 mL of LB broth containing 100 μg/mL of

ampicillin with the transformants of interest. The cultures were grown O/N in a 37°C shaker incubator set at 225 rpm. The next day the culture was poured into a 1.5 mL microfuge tube, spun down at maximum speed for 5 minutes, and the supernatent was poured off the cell pellet.

Midipreps were setup by inoculating 50 mL of LB broth containing 100 μg/mL of ampicillin with 1 mL of the miniprep culture of interest. The cultures were grown O/N in a 37°C shaker incubator set at 225 rpm. The next day the culture was poured into a 50 mL centrifuge tube, spun down at 10,000 g for 5 minutes at 4°C, and the supernatent was poured off the cell pellet.

DNA was isolated from the cell pellets by using commercially available kits, either the Wizard Plus Minipreps DNA Purification System or the Wizard Plus Midipreps DNA Purification System, both by Promega. Each kit consists of cell resuspension solution, cell lysis solution, neutralization solution, DNA purification resin, column wash solution, and the minipreps/midipreps columns. A vacuum manifold was used to allow for rapid processing of multiple samples. The protocol provided by the manufacturer was followed, producing consistent results.

RESULTS and DISCUSSION

CULTURES

Initial work with *B. bacilliformis*, KC583 and *B. henselae*, Houston in culture consisted of establishing the growth of the lyophilized bacteria obtained from ATTC on commercially available blood agar plates and chocolate agar plates. Once growth had been established on the solid media, the next step was to culture the organisms in a novel liquid media, modeled after the method used for culturing *Plasmodium falciparum*, another intraerythrocyitc parasite (see Methods and Materials). It was believed that the liquid media would more closely mimic the *Bartonella* ssp. natural intraerythrocytic environment, therefore making culture of the organisms faster and easier (1). A liquid, intraerythrocytic culture method was especially desirable for working with *B. bacilliformis*, since no animal model exists.

Growth was seen for *B. bacilliformis*, KC583 on both blood and chocolate agar plates, with the first discernible colonies seen on the chocolate agar plates after seven days. The chocolate agar plates contained lysed RBCs, as opposed to blood agar plates which contained intact RBCs,

which may have aided the growth of the organism. Growth was seen on the blood agar plates after ten days.

For setting up the liquid cultures, an isolated colony from the chocolate agar plate was picked. The organisms were resuspended in the liquid media, and then aliquoted to tissue culture plates. Growth was monitored both by the appearance of the culture and finding characteristic intraerythrocytic, pleomorphic bacteria within the RBC of a stained, thin blood smear. When *Bartonella* ssp. were present and growing in the liquid media, the color and appearance of the media would change from bright red with a RBC layer on the bottom of the plate and a clear supernatent, to brownish RBCs with a rusty colored supernatent and a dark precipitate. The changes in colors and the precipitate were from the RBCs being lysed as the bacteria multiplied within them. After the *Bartonella* ssp. had been maintained in culture for approximately a month, it was noted that numerous pleomorphic bacteria were found outside, as well as inside, the RBC. It was believed that the organisms were multiplying at a greater than expected rate in this culture system. The amount of infected RBCs in culture was decreased from 0.1 mL to 0.05mL at each subbing to maintain the once a week subculturing. More frequent subculturing was not desirable due to the lack of antibiotics in the liquid media (frequent handling would have increased the chance of contaminating the cultures).

Since no known *in vivo* animal existed for *B. bacilliformis*, it was decided to try various animal erythrocytes in culture to test their ability to support the organism. Guinea pig (gRBCs), pig (pRBCs), rabbit (rRBCs), and sheep (sRBCs) erythrocytes (Remmel) were substituted for hRBCs in the liquid culture media. The 8% suspensions of the animal erythrocytes in complete media were initially seeded with 0.1 mL of a mature (seven day old) *B. bacilliformis*, KC583 culture in hRBCs. After that, they were subcultured according to the basic protocol, with the exception being the different animal erythrocytes. Of the four animal RBCs used, sRBCs supported the growth of the *B. bacilliformis*, KC583 the best. On examination of Giemsa stained thin blood smears, numerous pleomorphic, intraerythrocytic bacteria were seen, and overall the culture and slides appeared very similar to what was seen with the organism in culture in hRBCs. The gRBCs, pRBCs, and rRBCs did not support the growth of the *B. bacilliformis*, KC583 as well. However, the problem may not have been that these animal RBCs were not suitable hosts for the bacteria, but that the animals' blood may have been old and/or contaminated. It was noted that upon receipt of the gRBCs, pRBCs, and rRBCs, the blood did not look fresh. All three were

a dark, reddish brown color, and when they were washed with RPMI 1640 before addition to the culture media, a high amount of lysis of the RBCs occurred. After two weeks, all cultures made with the gRBCs were contaminated. Most of the RBCs were lysed and the supernatent, in addition to being rusty colored from the RBCs' lysis, was cloudy and smelled foul. Examination of a Giemsa stained blood smear revealed numerous coccus-shaped bacteria in pairs and clusters in the media. The contaminate was not identified, and the cultures were destroyed. The pRBC and rRBC cultures never appeared to support the growth of the *B. bacilliformis*, KC583. Although the appearance of the cultures indicated RBC lysis, the blood had not looked healthy when it was introduced into culture, and examination of Giemsa stained blood smears revealed few pleomorphic, intraerythrocytic bacteria. After three weeks in culture, the pRBC and rRBC cultures were terminated due to the poor condition of the cultures and stock RBCs. *B. bacilliformis*, KC583 culture samples in sRBCs were frozen down for preservation due to the apparent success of the growth of the *Bartonella* within these erythrocytes. Before any definite conclusions on the growth of *B. bacilliformis*, KC583 in gRBCs, pRBCs, rRBCs, or sRBCs could be made, a better source of, and further work with, these RBCs in culture need to be performed.

The same basic protocol was followed for starting up *B. henselae*, Houston in culture as was used for *B. bacilliformis*, KC583. However, no growth was ever seen on the blood or chocolate agar plates. The plates were followed for four weeks before being disposed of. Since the organism did not grow on the solid media, it was decided to attempt to establish the organism in culture directly in the liquid media.

From the original PBS suspension of lyophilized *B. henselae*, Houston in PBS, 1.0 mL was added to the complete liquid media (exactly as if the culture was being subbed). The liquid cultures of *B. henselae*, Houston were subbed at 10% for the first two weeks and 5% for the next two weeks to establish the organism (as opposed to the normal 1%). After the first week, the appearance of the culture did not indicate any growth of the organism (the RBCs in the media were bright red and no precipitate was noted, indicating little lysis of the RBCs). Exam of a Giemsa stained blood smear, however, showed a few RBCs that contained pleomorphic, intraerythrocytic bacteria. By the fourth week (third subbing) in culture, the number of infected RBCs seen on Giemsa stained blood smears had increased, and it was noted that the liquid culture media had begun to turn brown (rusty looking) with a slight precipitate, indicating that RBCs in the culture were being lysed. However, a slight, milky-colored film was noted at this time on the surface of

the cultures, indicating possible contamination.

Initial plating of the *B. henselae*, Houston culture on nutrient agar plates did not show contamination, so it was believed that the problem was lipids from the FBS used in the media. A new batch of FBS was obtained, and the next four subbings were film-free, so batches of the *B. henselae*, Houston were frozen for preservation. When the frozen samples were brought back up into culture, the film reappeared. When nutrient agar plates were made this time, it was found that the cultures were contaminated with *Pseudomonas stutzeii*. All the components of the media (hRBCs, $NaHCO_3$, FBS, and RPMI 1640) were tested as possible sources of the contamination, and the FBS was shown to be contaminated with the same organism. At the same time, the *B. bacilliformis*, KC583 cultures came up positive for *Pseudomonas stutzeii*. It is uncertain at this time if any of the frozen samples of *B. henselae*, Houston in the lab are free from contamination with *Pseudomonas stutzeii*, and no further work has been done with the organism in culture.

PCR

A liquid media culture of *B. bacilliformis*, KC583 was used as the source of template DNA for all molecular studies involving this strain of *B. bacilliformis*. The DNA was harvested from a two day old culture to ensure that the bacteria were in the log phase of growth, and the *B. bacilliformis*, KC583's genomic DNA was isolated following the protocol described in the Methods and Materials section. Template DNA for *B. bacilliformis*, KC584 was obtained directly from Dr. M. Minnick in the form of a clone termed pKRT4. A miniprep procedure was used to obtain the DNA. Genomic DNA for the two clinical samples (4+ Sandi Conchop and LAB060) brought back from bartonellosis patients in Peru, was isolated following the protocol used for the culture strain of *B. bacilliformis*. There were two sources of the clinical sample DNA. The first was from the vacutainer blood sample from each of the patients, and the second was from one and two week old liquid cultures set-up on samples of the patients' blood.

The expected PCR product for *B. bacilliformis*, KC583 primer set 1 (amplicon 1) was 429 bp, based on the sequence information available on *B. bacilliformis*, KC584 (2,3). Following the protocol outlined in the Methods and Materials section for primer sets 1 and 2, the PCR product obtained for *B. bacilliformis*, KC583's amplicon 1 was found to be approximately 310 bp, based on agarose gel electrophoresis (**Figure**). This gave a difference of 119 bp between the expected and observed PCR products. The agarose gel of amplicon 1 showed that the PCR reaction was specific (one distinct band, with no smearing of the product, primer dimers, or nonspecific

amplification products present) (**Figure**), indicating that the conserved areas of homology in the intergenic spacer region that the primers recognized were intact, but that some differences were present in the variable region. *B. bacilliformis*, KC584 (the referent strain) did not yield an amplification product with primer set 1 due to a 5' deletion of 188 bp, which did not allow the sense primer to lay down, found in the *Sph* I clone of the intergenic spacer region (3).

The expected PCR product for *B. bacilliformis*, KC583 primer set 2 (amplicon 2) was 497 bp (2,3). The major PCR product obtained for *B. bacilliformis*, KC583's amplicon 2 was found to be approximately 600 bp, based on agarose gel electrophoresis (**Figure**). This gave a difference of 103 bp between the expected and observed PCR products. The agarose gel of amplicon 2 showed the presence of multiple bands, with the major band falling in at 600 bp, indicating that the PCR reaction was not specific (some smearing, but no primer dimers) (**Figure**). The nonspecificity of the reaction appeared to be due to the primers not laying down as expected. The antisense primer for primer set 1 and the sense primer of primer set 2 were both designed (see Methods and Materials) to recognize the same conserved area of the intergenic spacer region and since the primers for amplicon 1 yielded a specific product, it was concluded that the conserved region the antisense primer of primer set 2 recognized was either deleted or had a different sequence. When primer set 2 was used to generate a PCR product using *B. bacilliformis*, KC584 as the template, a specific product of the expected size (497 bp) was obtained (**Figure**), giving further evidence of the sequence differences between *B. bacilliformis*, strains KC583 and KC584.

The expected PCR product for *B. bacilliformis*, KC583 primer set 3 (amplicon 3) was 1.75 kilobases (kb), based on the sequence information available on *B. bacilliformis*, KC584 (2,3). Following the protocol outlined in the Methods and Materials section for primer set 3, the PCR product obtained for *B. bacilliformis*, KC583's amplicon 3 was found to be approximately 1.2 kb, based on agarose gel electrophoresis (**Figure**). This gave a difference of approximately 500 bp between the expected and observed PCR products. The agarose gel of amplicon 3 showed that the PCR reaction was specific (one distinct band, with no smearing of the product, primer dimers, or nonspecific amplification products present) (**Figure**), indicating that the conserved areas of homology in the 16S and 23S rRNA genes that the primers recognized were intact, but that sequence variation was present within the amplified region. *B. bacilliformis*, KC584 (the referent strain) did not yield an amplification product with primer set 3 since the *Sph* I clone was only of the intergenic spacer region and it did not contain the 5' 16S and 3' 23S rRNA gene sequences the

the sense and antisense primers recognized (2,3).

Since it was unknown how the two clinical samples of *B. bacilliformis* (4+ Sandi Conchop and LAB060) would compare to the two strains (KC583 and KC584) available in the lab, it was decided to use primer set 3 for PCR of the DNA extracted from the clinical samples. It was believed that a product would be obtained using these primers since they were designed to lay down in conserved areas of the *B. bacilliformis* genome (see Methods and Materials). Genomic DNA extracted from the patients' blood samples (not from cultured material) was used as template. Following the protocol outlined in the Methods and Materials section for primer set 3, PCR reactions with template concentrations of 10 ng, 50 ng, 100 ng, and 250 ng were setup for each of the clinical samples. Agarose gel electrophoresis of the PCR amplification products showed amplification only for the 4+ Sandi Conchop sample. The products were approximately 1.7 kb in size, the same as the referent strain *B. bacilliformis*, KC584 (2,3). Based on the size of amplification products run out on agarose gel, it appeared that this clinical sample was more closely related to *B. bacilliformis*, KC584, then to the other strain in the lab, *B. bacilliformis*, KC583. All four template concentrations gave specific (one distinct band, with no smearing of the product, primer dimers, or nonspecific amplification products present) product. Giemsa stained thin smears of the patient's blood revealed > 90% of the RBCs infected, so obtaining sufficient DNA from the blood sample to serve as template had not been difficult.

No PCR product was obtained for the LAB060 clinical sample. The gel of the amplification products showed non-specific, low-molecular weight bands and primer dimers for all four template concentrations. A possible explanation for the lack of amplification with this patient's blood was that they had received chloramphenicol for approximately one week prior to their blood being drawn. The antibiotic therapy may have reduced, or possibly eliminated, the *B. bacilliformis* in the patient's blood to a level that was not sufficient for isolation and amplification using the above protocol.

RFLP

Since the PCR products of primer sets 1 and 2 were not of the expected sizes on agarose gel electrophoresis, it was decided to further analyze the amplicons using RE digestion to produce RFLP maps (see Methods and Materials). PCR-based RFLP has been used to differentiate between species and for species subtyping, so it was believed that this strategy would give further details on the apparent strain variation seen between the two *B. bacilliformis* ssp. used in the lab

(4). REs that cut rarely (preferably once or twice to produce two or three fragments) within the amplicons were chosen. The PCR products of amplicon 1 and 2 were digested with the REs and analyzed using agarose gel electrophoresis. The RFLP results are shown in **Tables 1** and **2.**

Amplicon 1 of *B. bacilliformis*, KC583 was cut by the RE's in **Table 1,** but did not yield fragments of the expected lengths (**Figure**) (2,3). The RFLP map of the RE digest of amplicon 1, seen by agarose gel electrophoresis (**Figure**), was not consistent with the expected appearance of the digested PCR product. Since the PCR product of primer set 1, amplicon 1, was not the predicted size (**Figure**), it was expected that the RFLP products would vary. It is believed that the REs used cut in conserved regions since they cut in *B. bacilliformis*, KC583, and it was believed that the differences between amplicon 1 of *B. bacilliformis*, KC583 and *B. bacilliformis*, KC584 were in variable regions (due to the specificity of the PCR product obtained). As mentioned above, amplicon 1 of *B. bacilliformis*, KC584 was not available for comparison RE digests due to the 5', 188 bp deletion found in the *Sph* I referent clone.

Amplicon 2 of *B. bacilliformis*, KC583 was not cut by the RE's in **Table 2.** The RFLP map seen on the agarose gel electrophoresis of the RE digested amplicon 2 was consistent with the appearance of the undigested PCR product of amplicon 2 (multiple bands were seen in the same areas of the gel; compare **Figure** with **Figure**). Amplicon 2 of *B. bacilliformis*, KC584, however, did cut with the RE's, and gave the expected fragment lengths (**Table 2, Figure**) (2,3). RFLP analysis supported the conclusion that the area of the intergenic spacer region amplified by primer set 2 for *B. bacilliformis*, KC583 differed from that of the referent strain, *B. bacilliformis*, KC584 (probably in a conserved area) due to the lack of digestion of the nonspecific PCR product.

SEQUENCING

Based on the apparent variations between the two strains of *B. bacilliformis* in the lab, it was decided to sequence the PCR products of *B. bacilliformis*, KC583. Before sequencing could be done, it needed to be determined if primer set 1 and 2 would amplify their PCR products, amplicon 1 and 2. A 1:10 dilution of amplicon 1 and 2 were used as template DNA for PCR reactions setup according to the protocol previously outlined in the Methods and Materials. Specific PCR products (single bands), as seen on agarose gel electrophoresis, were produced by primer set 1 and 2 when amplicon 1 and 2 were used as the template DNA. Since the existing primers would work to amplify their specific PCR product, new, internal primers did not have to

TABLE 1:

RE, Cut Points, and Expected and Observed Fragment Lengths for Amplicon 1.

		Fragment Lengths (in bp)	
Enzyme	Cuts at bp	Expected	Observed
Bam HI	84	68, 355	310
Bsp MI	41	25, 398	280
Hpa II	157, 356, 371, 381	141, 99, 15, 10, 58	125

Note: Expected fragment lengths and enzyme cut points were obtained from DNA Strider based on sequence information from GeneBank accession L26364.

TABLE 2:

RE, Cut Points, and Expected and Observed Fragment Lengths for Amplicon 2.

		Fragment Lengths (in bp)	
Enzyme	Cuts at bp	Expected	Observed
Aat II	876	461, 36	no cut
Blp 1	593	187, 310	no cut
Bsp MI	610	204, 293	no cut
Hpa II	649	243, 254	no cut
Xho I	884	19, 478	no cut

Note: Expected fragment lengths and enzyme cut points were obtained from DNA Strider based on sequence information from GeneBank accession L26364.

be designed for the ABI Prism Dye Terminator Cycle Sequencing Core Kit that was going to be used to generate sequencing data (see Methods and Materials).

Since sequencing of a PCR product could be difficult (i.e., residual contaminates from the PCR reaction used to generate the sequence of interest or secondary structure of the sequence could cause interference) it was decided to attempt to sequence amplicon 2 first. The area of *B. bacilliformis*, KC583 amplified by primer set 2 appeared to vary more from the published sequence (*B. bacilliformis*, KC584) than the amplicons produced by primer sets 1 and 3, so it was believed that if the cycle sequencing protocol worked for amplicon 2, it would work for the others. The first sequencing run on amplicon 2 did not work. While the control reaction using pBS worked (indicating that the reagents were sound), the gel of the sequencing product showed nothing. It appeared that not enough template had been used for the reaction, and further investigation showed that approximately thirteen times more primer was used than necessary. A second sequencing run was performed, with the DNA template concentration being increased from 50 ng/μL to 100 ng/μL, and the amount of primer used decreased from 8 μM/100μL to 0.6 μM/100μL. The second sequencing reaction also did not produce the desired results. Both the sense and the antisense primers of primer set 2 laid down on their templates and extension of the sequencing product initiated, but after the first 10-20 bp, the primers fell off and extension was terminated. No ready explanation for this could be determined. Further sequencing of the PCR products was terminated, and it was decided to attempt to clone the area of *B. bacilliformis*, KC583 we were interested in to generate large amounts of clean DNA that could than serve as template for sequencing.

CLONING

Obtaining the DNA of interest for sequencing through cloning (ligation of the DNA into a plasmid vector) would give DNA that was better suited for cycle sequencing than DNA obtained through PCR. The DNA of interest would be produced by a plasmid within transformed cells. Many of the currently used plasmid vectors (e.g., those of the pUC series, like pBS) replicate to such a high copy number that they can be purified in large quantities from cultures that have simply been grown to late log phase in standard LB medium (5,6). Extraction and clean-up of the DNA using commercial mini- or midiprep kits would be fast and efficient. Primer set 3 was designed to produce amplicon 3 for the purpose of cloning (see Methods and Materials). Amplicon 3 contains

the entire intergenic spacer region, including both the areas amplified by primer set 1 and 2 **(Figure)**.

For cloning, it was decided to use pBS phagemids (plasmids with a phage orientation) for a number of reasons. First, the plasmid and a line of competent cells it could be transformed into, were readily available in the lab. Second, pBS contains the β-galactosidase gene which allows for rapid blue/white screening upon transformation of the appropriate competent cells. The polylinker site and T7 and T3 RNA polymerase promoter sequences are present in the N-terminal portion of a *lac*Z gene fragment. pBS phagemids that do not contain an insert in the polylinker area grow as blue colonies on the appropriate strains of bacteria. pBS that contains the insert will grow as white colonies on the same strain since the insert disrupts the coding region of the *lac*Z gene fragment. Finally, since pBS phagemids replicate autonomously as plasmids, colonies (not plaques) are obtained following transformation (5,6).

MAX Efficiency DH5α Competent Cells were used for transformations due to their φ80d*lac*ZΔM15 marker which provides α-complementation of the β-galactosidase gene from pUC or similar (pBS) vectors. This complementation allows for blue/white screening of colonies on LB agar plates containing ampicillin, X-gal, and IPTG (see Methods and Materials). Colonies containing phagemids without inserts will be blue, while colonies with phagemids containing inserts will remain white, after incubation for 12-18 hours at 37°C. DH5α cells are also capable of being transformed efficiently with large plasmids and consistently yield high transformants, with or without saturating amounts of DNA, making them easy to work with (GibcoBRL).

Plasmids are usually purified from liquid cultures (grown in medium containing the appropriate antibiotic) that have been inoculated with a single bacterial colony picked from an agar plate. Most of the commonly used methods for plasmid purification exploit the relatively small size and covalently closed circular nature of plasmid DNAs, and involve three basic steps: growth of the bacterial culture, harvesting and lysis of the bacteria, and purification of the plasmid DNA. For the purpose of reproducibility, commercial mini- and midiprep kits were used to purify the plasmid DNA from bacteria. Since the kits did not require an organic extraction or ethanol precipitation (both steps that could leave contaminants with the DNA that could interfere with further manipulation), the purified plasmid could be used directly for automated fluorescent DNA

sequencing.

Early ligation work with the plasmid vector and insert (amplicon 3) was discouraging (see Methods and Materials). The original stock of pBS used was a lab dilution that had been in storage (-20°C) for over a year, and its exact condition was unknown. The starting concentration of the stock pBS was 0.8 μg/mL, but it was estimated to have dropped to 0.4 μg/mL after RE digestion and clean-up of the DNA (using the DE81 paper method). After RE digestion and clean-up of the insert DNA (using the DE81 paper method), concentration of the original stock solution of insert was difficult to determine, but estimated to be 1.5 μg/mL. For ligation, the ideal ratio of insert-to-vector DNA is variable, however, the usual starting point is 2:1, insert to vector, measured in available picomole ends for ligation (6). The calculation for determining this is: picomole ends/mg of DNA = (2×10^6)/(number of bp x 660). For the first ligation, 0.01 μg/mL of insert was used to 0.001 μg/mL of pBS, a ratio of 10:1, since the quality of the insert DNA was questionable. The ligation mixture was transformed into DH5α cells (see Methods and Materials), and the transformation mix plated out. A control ligation consisting of the double RE digested pBS that had been ligated back onto itself was used to check the efficiency of the transformation (since the control vector ligation had no insert in the polylinker site, the pBS would grow as blue colonies on the appropriate strains of bacteria). Single, isolated white bacterial colonies were picked using a sterile toothpick and inoculated into LB broth to setup a miniprep culture. The pBS was purified using a miniprep kit (see Methods and Materials) and analyzed for the presence of the insert in the plasmid by double RE digestion with *Eco* RI and *Hind* III. The digestion was run out using agarose gel electrophoresis. A successful ligation/transformation/plasmid recovery would have been marked by the presence of a 1.2 kb band for the insert, and a 3.1 kb band for the plasmid. The digest of the first miniprep showed only the presence of the pBS (a 3.1 kb band in the gel). The remainder of the ligation mixture was used for a new transformation reaction, but again, the RE digestion of the miniprep showed only the 3.1 kb pBS band.

Closer examination of the gel containing the miniprep digestions from the second transformation showed a very faint band approximately in the 1.2 kb range. To determine if this faint band may have been the insert, three single RE digestions with *Eco* RI, *Hind* III, and *Bgl* I

were performed. If the insert was present, the following bands would have been visible in the gel: *Eco* RI and *Hind* III would each give a band approximately 4.6 kb, and *Bgl* I a band 3.9 kb. The agarose gel of the digestion showed bands of 3.1 kb for the *Eco* RI and *Hind* III, and a band of 1.5 kb for the *Bgl* I, indicating that the insert was not present in the vector. A possible explanation for this was that the pBS and insert may not have been fully digested during the double *Eco* RI and *Hind* III digestion that was performed before ligation for the generation of complementary sticky ends. It was decided to digest the pBS and insert each with two single RE digestions (as opposed to one double) in the hope of improving the action of each enzyme. If the previous double RE digestions had been incomplete, than the available picomoles of complementary sticky ends needed for successful ligation would not have been present. It was also decided to generate more insert (PCR of amplicon 3) and to clean it up using the Wizard kit to produce insert of a higher concentration, making it easier to use for ligations.

Insert and pBS were redigested with two single RE digestions. Although the insert and pBS were singly digested with the *Eco* RI and *Hind* III, the exact concentration of available picomole ends was still unknown. Agarose gel electrophoresis would not be able to quantitate how completely the REs had cut since only a few bp on each end were being digested away. Three ligation reactions, with the concentration of insert to plasmid being 5 ng:10 ng (1:2), 5 ng:2.5 ng (2:1), and 5 ng:0.25 ng (1:0.25), were setup to compensate for the lack of exact data on the concentration of available sticky ends for the ligation. Three transformation reactions using the three ligation mixtures were performed, but few colonies were produced, with only several being white. The white colonies were picked, analyzed using minipreps, and were not positive for the insert (i.e., successful ligation). The three ligation reactions (ratios) were repeated using the new, more concentrated insert (generated using primer set 3), that had been digested singly with each of the REs. The transformations and minipreps were repeated, but once again without success.

Since so few total (blue and white) colonies were being produced, it was thought that something was going wrong with the actual transformation process. The transformation protocol for the DH5α cells was modified in an attempt to remedy the situation. The cells were thawed on wet ice (as opposed to dry) and the transformation reactions were performed in pre-chilled 17 x 100 mm polypropylene tubes (instead of microfuge tubes). The ligation mixtures were further diluted before the transformation reaction to ensure that the total DNA concentration did not exceed

the limit for maximum transformation efficiency. Finally, the cells were heat-shocked for 45 seconds instead of 2 minutes, and care was taken not to shake the cells up during the process.

A new ligation reaction was setup, with the ration of insert to plasmid being 5 ng:10 ng (1:2). More pBS than insert was used because the concentration and condition of the insert was better known (the insert had just been generated through PCR using primer set 3). The exact condition and concentration of the plasmid was questionable since the stock being used was old (approximately two years). The modified protocol for transformation of the DH5α cells was followed. Numerous colonies were produced, indicating that the modified protocol improved the transformation efficiency. However, few of the colonies were white, and none of the white colonies that were picked and analyzed using minipreps showed the presence of the insert. Transformation had been improved, but the ligation reaction was still not successful.

One possible explanation for the presence of so many blue colonies, and so few white, was that the pBS had not been completely digested by the *Eco* RI and *Hind* III. To explore this possibility, it was decided to redigest the stock of pBS being used overnight with the two REs to ensure complete digestion. To check the RE digestion, control ligations of undigested, suspected partially digested, and the overnight digested plasmid were performed. If the pBS was partially digested (i.e., the plasmid was cut with one RE, not both, or the RE only partially cut and therefore did not digest completely) it would ligate back onto itself and transform the DH5α cells to give blue colonies. If the pBS was properly digested it would not have complementary ends, and therefore would not ligate back together. Since the cells like to take up circular DNA as opposed to linear, the unligated plasmid would not successfully transform into the DH5α cells. As a result, the DH5α cells would not grow on LB Agar with ampicillin since the pBS would not be present in the cells to confer antibiotic resistance. The control ligations were transformed into HB101 cells since there were no DH5α cells available. The HB101 cells used were not capable of blue/white screening, but they would take up the pBS if it had ligated back onto itself. Since the plasmid conveyed the antibiotic resistance necessary for growth on the ampicillin plates, and only undigested or partially digested pBS (that had ligated back onto itself) would transform the cells, the presence of any colonies on the plates would suggest that the pBS was not properly digested. No colonies were seen on any of the HB101 cells' plates. It was expected that colonies would at

least have been seen on the plate that contained the cells that had been transformed with the undigested pBS. However, since the HB101 cells used were old and may not have been viable, it was decided to repeat the pBS control ligations using a fresh batch of DH5α cells. When the control ligations were repeated with the new DH5α cells, numerous blue colonies were seen on all the plates (undigested, partially digested, and overnight digested), indicating that there was still something wrong with the plasmid.

To further characterize the pBS, it was decided to perform several RE digestions with enzymes that cut in unique areas of the plasmid (outside of the multiple RE cloning site) or insert. Two enzymes were chosen, *Bgl* I and *Ava* I (expected fragment lengths were obtained from DNA Strider based on sequence information from GeneBank accession L26364) (2). pBS that had been ligated with the insert and pBS without the insert were each digested. *Bgl* I was expected to cut in two places in the pBS to generate two fragments. A 1.58 kb fragment should have been generated regardless of the insert; if the insert was present, a 1.58 kb and a 2.77 kb band should have been seen; if just the plasmid was present, two 1.58 kb bands (which would have appeared as one large band on the gel) would have been seen on agarose gel electrophoresis analysis of the digestion. *Ava* I was expected to cut in two places in the insert to generate two fragments. If no insert was present with the pBS (either the pBS had not been ligated to the insert, or the ligation was unsuccessful), than the plasmid would not have been digested and would have migrated in the gel as undigested, circular plasmid. If the insert had been successfully ligated with the pBS, than two fragments would have been generated, one 57 bp and the other 4.3 kb, but only the 4.3 kb band would be visible in the gel. If digestion of the insert had occurred, the plasmid with the insert would have been linearized and migrated as such in the gel allowing for detection of a successful digestion. Neither the *Ava* I or the *Bgl* I digestions turned out as predicted. *Ava* I did not digest at all. This would be expected for the digestion of the pBS alone, or with pBS that had not been ligated with the insert. However, *Bgl* I also did not digest. This was unexpected since the *Bgl* I cut within the pBS, regardless of the insert, outside of the multiple cloning site. The *Bgl* I digested pBS looked like undigested, circular plasmid on the agarose gel. The *Bgl* I digestions of the plasmid suggested that the pBS was somehow altered, and that it had lost RE sites. It was decided that the stock of pBS that had been used for all ligations up to this point was altered and therefore not performing as expected. This stock of pBS was discarded, and new pBS was ordered from

Stratagene.

New stocks of insert and pBS were each singly digested with both *Eco* RI and *Hind* III in preparation for ligation. The ratio for the ligation reaction of the insert to the new plasmid was 56.7 ng of insert: 17.4 ng of pBS (3.25:1). A control ligation using the pBS alone was performed to check the efficiency of the RE digestions and transformation. The ligation mixture was transformed into DH5α cells, using the modified protocol, and the cells were plated out. All of the transformation plates had isolated colonies after O/N incubation. The control plates had no white colonies, indicating that the RE digests of the pBS were successful, while the plates that had the pBS with insert had numerous isolated white colonies scattered among the blue ones. Fourteen white colonies were picked using a sterile toothpick to setup for minipreps. *Eco* RI and *Hind* III digestion of the minipreps revealed one miniprep (miniprep 1) that looked like it might contain the insert (the gel showed two bands, one at 1.2 kb for the insert, and one at 3.1 kb for the plasmid). A midiprep (midiprep 1) of miniprep 1 was setup to further investigate this clone. A midiprep of the clone would produce larger quantities of cleaner DNA (less protein contamination from the extraction/clean-up procedure) for further manipulation. The agarose gel of the *Eco* RI and *Hind* III digestion of midiprep 1's DNA looked similar to that of the gel for miniprep 1. To investigate the clone more fully, it was decided to digest midiprep 1 with several REs to generate a RFLP map.

Ava I, *Bam* HI, *Bgl* I, *Bsp* MI, and *Eco* RI were chosen for the RE digestions of midiprep 1. *Ava* I and *Bsp* MI both cut twice in the insert, *Bam* HI cut once in the insert, *Bgl* I cut twice in the pBS, and *Eco* RI cut once at the ligation point of the insert to the pBS. Controls for midiprep 1 DNA digestions (the pBS with the insert) were insert DNA that was digested with *Ava* I, *Bam* HI, and *Bsp* MI. Undigested midiprep and insert DNA were run out on the gel with the digested DNA for comparison. The results of the RFLP analysis of midiprep 1 are shown in **Table 3.**

The RFLP analysis of midiprep 1 revealed several things. First, the only enzymes that cut as expected were those that cut in the pBS, or at the junction of the insert and plasmid (*Bgl* I and *Eco* RI). This indicated that the pBS was intact (it contained its expected RE sites), and that the *Eco* RI ligation site was present in the plasmid and insert. The *Eco* RI digestion linearized the midiprep DNA and produced a band in the agarose gel that fell in at approximately 4.3 kb. This would be expected for midiprep DNA that was the pBS ligated with the insert. The *Bgl* I digestion

TABLE 3:

RE and Expected and Observed Fragment Lengths for MidiPrep 1.

	Cuts at bp		Fragment Lengths (in bp)	
Enzyme	Insert	pBS	Expected	Observed
Ava I	96, 153	-----	57, 4296	no cut
Bam HI	85	-----	4300	no cut
Bgl I	-----	709, 2337	1576, 2777	1576, 2777
Bsp MI	42, 610	-----	568, 3732	no cut
Eco RI	881	-----	4300	4300

Note: Expected fragment lengths and enzyme cut points were obtained from DNA Strider based on sequence information from GeneBank accessions M65249 (16S rRNA gene), L26364 (intergenic spacer region), and L39095 (23S ribosomal gene) (2).

of the midiprep produced the two bands expected (1.57 and 2.77 kb) for DNA that was the pBS ligated with the insert.

The bands seen on the agarose gel electrophoresis of the *Ava* I, *Bam* HI, and *Bsp* MI digested midiprep and insert DNA were consistent with the appearance of the undigested midiprep and insert DNA. This suggested that these REs were not able to cut within the insert. The data that was used to predict where these enzymes would cut in the insert was based on sequence information for *B. bacilliformis*, KC584. Since the insert DNA was generated through PCR using *B. bacilliformis*, KC583 as template, and earlier PCR work on *B. bacilliformis*, KC583 showed that it differed from KC584, the results seen in the digestion were not unexpected. Of particular interest was the lack of digestion by *Bsp* MI. *Bsp* MI was predicted to cut once in the 16S rRNA gene at the beginning of the insert, and again in the gene for the tRNA Ala in the insert. These cut sites were predicted using sequence information for *B. bacilliformis*, KC584. Both of these areas are conserved areas of the spacer insert, but they were either absent or altered in the insert DNA of *B. bacilliformis*, KC583. It is also interesting to note that primer set 1 produced specific PCR product. Primer set 1's sense primer laid down in the 16S rRNA gene, so this area's sequence probably does not differ that much between the two *Bartonella* ssp. However, primer set 2's antisense primer was designed to lay down in the tRNA Ala area, and since primer set 2's PCR product was not that specific, and the REs that were predicted to cut in this area did not, it was concluded that this area differs between *B. bacilliformis*, KC583 and KC584. Although the enzymes that were chosen to cut in the insert did not, the fragment lengths obtained from the two enzymes that cut in the plasmid support the conclusion that a clone containing the pBS with the PCR product insert was produced.

FUTURE WORK

Although the data obtained from the work of culturing the *Bartonella* ssp. *in vitro* using a system modeled after that for culturing malaria parasites was positive, additional work to answer questions that arose needs to be performed. The main area that requires further investigation deals with culturing the *B. bacilliformis*, KC583 in different animal erythrocytes, in an attempt to help answer questions dealing with the natural (or potential) host(s) for *B. bacilliformis*. Aside from the sRBCs, better sources of gRBCs, pRBCs, and rRBCs need to be obtained. It would also be interesting to try to culture the *B. bacilliformis*, KC583 in domestic cat RBCs, if a source could be

found. Also, culturing in the different animal erythrocytes should be done with *B. henselae*, Houston to see if it has the potential to survive in the various RBCs. Electron microscopy of the *Bartonella* ssp. would also provide valuable insights into the behavior of the organisms in this culture system. Freeze fracture sections of infected erythrocytes could show the *Bartonella* ssp. within the RBC, supporting the belief that the organism invades the red cell in this culture model. Work also needs to be done on designing a transport media to keep *B. bacilliformis* collected from a patient in the field viable until it could be brought back to the lab and setup in culture.

PCR and RFLP data raised a number of areas for further investigation. The data obtained suggested sequence variation between the lab strain of *Bartonella, B. bacilliformis*, KC583, and the referent strain *B. bacilliformis*, KC584. Sequencing of the area of interest, the 16S-23S rRNA intergenic spacer region of the *B. bacilliformis*, KC583, would determine the sequence variations between the two strains. Since sequencing of the PCR product was not successful, the development of a clone containing a plasmid with the insert ligated into it became a priority. Current data suggests that a successful clone has been produced, but additional work needs to be done to confirm this. To confirm that the clone contains the *B. bacilliformis*, KC583 insert, and that the DNA produced with the three primer sets is *B. bacilliformis* DNA (not a contaminant), southern hybridization should be done. The PCR product of pKRT4 amplicon 2 and the intact pKRT4 plasmid would each be labeled to use as probe. The pKRT4 clone was obtained from Dr. M. Minnick, so it is a known to be *B. bacilliformis*, KC584. Although there does appear to be sequence variation between the two strains, there should be more than enough homology between the sequences for southern hybridization to confirm the presence of *B. bacilliformis*.

In addition to the southern hybridization to confirm the presence of *B. bacilliformis* DNA, nested PCR should be done on all samples (culture *B. bacilliformis*, KC583 and the clinical samples, 4+ Sandi Conchop and LAB060) to increase the sensitivity and specificity of the PCR reactions performed on extracted genomic DNA. Primer set 3 would be used to amplify the entire intergenic spacer region, including parts of the 16S and 23S rRNA genes, from the genomic DNA. The PCR product obtained from primer set 3 would then be used as template for additional PCR reactions using primer sets 1 and 2. This type of nested PCR would give an increase in the specificity of the product and an increase in sensitivity from using the lower copy number product of primer set 3 (as opposed to the genomic DNA of the whole organism) as the template.

Finally, controls for the PCR reactions need to be developed. PCR reactions using all three

primer sets needs to be performed on a variety of organisms to determine if the primers will amplify sequences from non-*Bartonella* ssp., in addition to determining how the PCR products of these primers differ among *Bartonella* ssp. PCR using primer sets 1, 2, and 3 needs to be done on the *B. bacilliformis*, Houston present in the lab to ascertain what amplification products would be produced. Also, as many different *Bartonella* ssp. and strains as possible should be collected and amplified using these primers so that the products could be compared. *Bartonella* ssp. are members of α-2 subgroup of the class *Proteobacteria*, so amplification using primer sets 1, 2, and 3 should be performed on as many other members of this class (such as *Rickettsia* ssp., *Erlichia risticii*, *Brucella abortus*, and *Agrobacterium tumefaciens*) as possible to determine what, if any, amplicons are produced. Also, some of the more common environmental bacteria (such as *Streptococcus* ssp., *Staphylococcus* ssp., and *Pseudomonas* ssp.) should be subjected to amplification to determine if any product is produced from the primers. If these bacteria produce amplicons with any of the primer sets, then the sizes and RFLP patterns of the product should be noted to use as comparison controls when amplifying suspected *B. bacilliformis* DNA.

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METHODS and MATERIALS

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RESULTS and DISCUSSION

1. Wong MT, Thornton DC, Kennedy RC, and Dolan MJ. A chemically defined liquid medium that supports primary isolation of *Rochalimaea henselae* from blood and tissue specimens. *J. Clin. Micro.* 1995. 33(3): 742-744.
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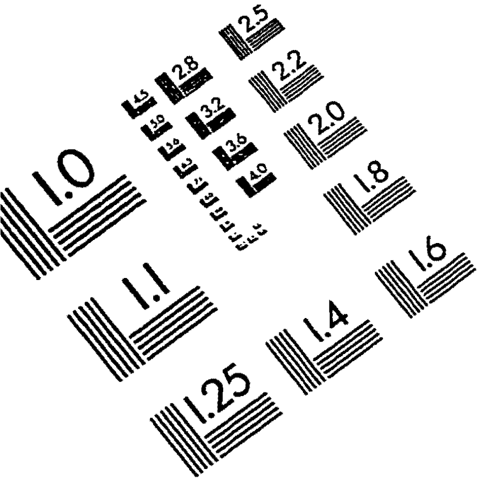
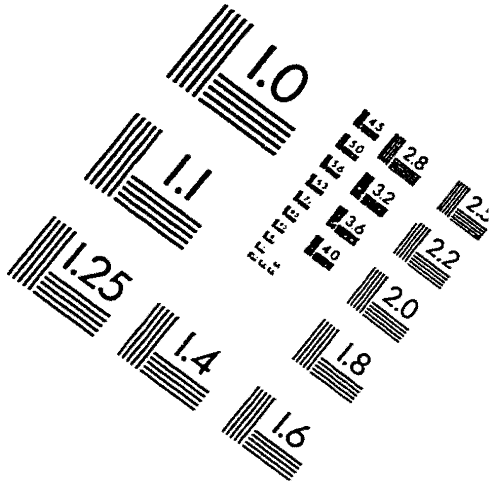
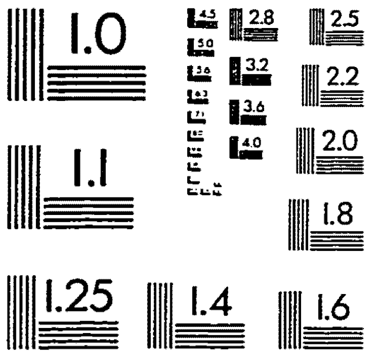
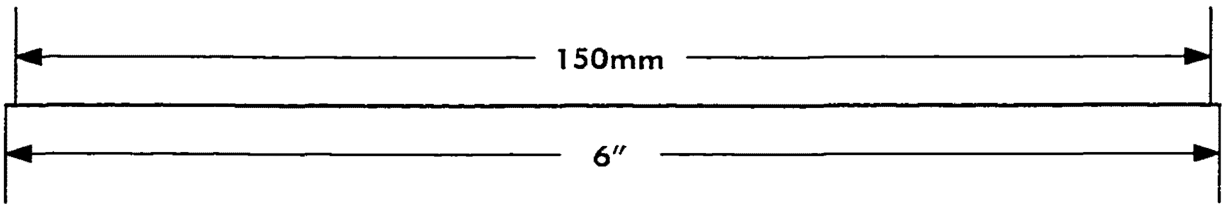
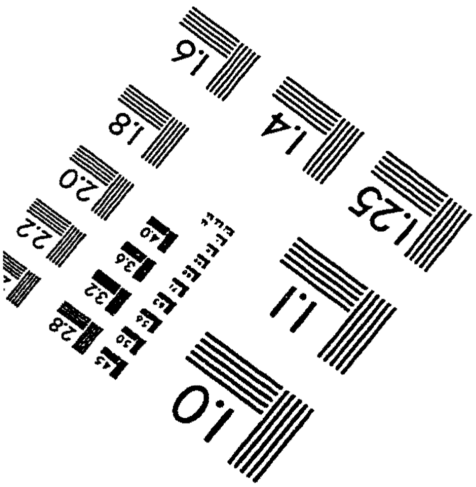
IMAGE EVALUATION TEST TARGET (QA-3)

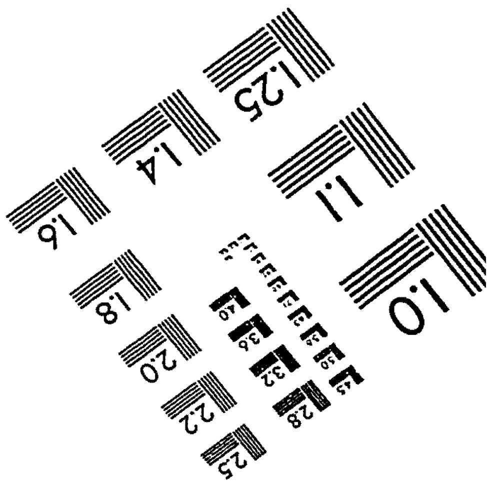












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