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I am submitting herewith a thesis written by Wallace H. Greene entitled "The Influence of Pili on the Attachment of Moraxella Bovis to Bovine Cornea as seen with Scanning Electron Microscopy." I recommend that it be accepted in partial fulfillment of the requirements for the degree of Master of Science, with a major in Microbiology.

John M. Woodward

John M. Woodward, Major Professor

We have read this thesis
and recommend its acceptance:

David A Bemis

Paul C. Smith DVM

John W. Allen, DVM

Accepted for the Council:

L Evans Roth

Vice President
Graduate Studies and Research

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THE INFLUENCE OF PILI ON THE ATTACHMENT OF *MORAXELLA BOVIS* TO BOVINE CORNEA AS SEEN WITH SCANNING ELECTRON MICROSCOPY

A Thesis

Presented for the

Master of Science

Degree

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Wallace H. Greene

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W.H.G.

ABSTRACT

The purpose of this study was to more clearly define the role pili play in the attachment of Moraxella bovis to corneal tissue. By treating freshly collected cornea in various ways and examining the results with the techniques of scanning electron microscopy, it was possible to obtain a more accurate picture of the pathogenic process.

It was found there is a significant difference in the number of bacteria that attach to bovine corneal tissue when piliated vs. nonpiliated bacteria were used. Pretreating the bacterial cells by heating them severely damaged their pili and greatly reduced their attachment. Pretreatment with formalin, however, had no effect on the pili or the attachment of the bacteria to the corneal epithelial tissue. No attachment was seen when rabbit and canine cornea were examined under identical conditions. Finally, pretreatment of the bovine cornea with purified pili before exposure to the organism significantly reduced the number of attaching bacteria.

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CHAPTER I

INTRODUCTION

Infectious bovine keratoconjunctivitis (IBK) is a common disease of cattle and is found throughout the world (43). It is highly contagious, and all breeds of cattle are susceptible (32). Outbreaks of IBK can occur any time during the year, but are more common during the summer months when contributing factors such as fly populations and solar radiation are higher (11,32).

IBK is a significant disease due to its effect on the cattle industry (2,32). The disease causes economic loss due to beef cattle having a reduced rate of weight gain or a loss of weight. Milk production may be reduced by 25% or more in dairy cows. There are also treatment costs and reduced sale prices due to eye disfigurements and blindness. In a study done in 1975 on purebred Hereford cattle (20), infected calves were reported to have a suppression of weight gain, in many cases exceeding 30 pounds. At 1975 beef prices, this was estimated at a loss of 12 to 14 dollars per animal. In a more recent survey taken by Dr. Ralph Hall of The University of Tennessee Agriculture Extension Service, the average loss per affected animal in Tennessee herds in 1977 was 29 dollars. It was estimated that 32% of the one million calves produced that year had IBK. This totals to a loss of ten million dollars for Tennessee cattlemen for that year alone. The United States Department of Agriculture estimated a national loss of one hundred sixty-two million dollars in 1976 for the entire country.

Moraxella bovis, Moraxella liquefaciens, Moraxella nonliquefaciens, Neisseria catarrhalis and Acinetobacter are all commonly isolated from the bovine eye (45). Moraxella bovis is the only organism recovered from the bovine eye for which one can fulfill all of Kochs postulates (8,13,43,44). M. bovis and N. catarrhalis are found more frequently in animals having IBK than in normal animals (44). Of these organisms, M. bovis is the only one which shows any significant seasonal variation in incidence.

Hemolytic M. bovis is the predominant isolate from cattle having acute IBK (1,25,30). The most virulent colonies are flat, firm, agar-corroding and tend to autoagglutinate when suspended in liquids. Nonhemolytic and hemolytic colonies, which do not autoagglutinate, do not appear to be pathogenic. In one report (26), hemolytic M. bovis isolated from a naturally occurring case of IBK produced hemolytic and nonhemolytic variants when subcultured. Eyes were then exposed to the nonhemolytic variants and became infected, however, no disease was produced. When the animals were reexposed to the hemolytic variants, IBK was produced in 30 out of 34 eyes. The nonhemolytic M. bovis was also reported to produce hemolytic and nonhemolytic progeny on blood agar plates.

M. bovis can be identified by the following characteristics (25). It is a gram negative, nonmotile diplobacillus, and usually hemolytic. It does not reduce nitrates to nitrites, ferment carbohydrates or grow on Herellea agar. It is oxidase positive and proteolytic, producing a three zone reaction in litmus milk. Catalase positive and negative strains of hemolytic M. bovis are capable of producing IBK (9).

Hemolytic M. bovis can be found established in the eyes and nasal cavities after experimental infection of the eyes. As few as one thousand organisms suspended in 10% magnesium chloride can induce IBK (30). Organisms can be recovered after infection for 2 to 72 days (10). Average recovery days for three separate experiments were 24, 25 and 27 days. The keratitis, or corneal inflammation, is thought to be a sequel to severe conjunctivitis in IBK. In one report (26), conjunctivitis was seen without the appearance of keratitis in 21 eyes. Conjunctivitis preceded keratitis in an additional 21 eyes.

M. bovis can be induced to cause disease by such influences as other organisms and ultraviolet irradiation (32). Ultraviolet radiation may be a primary etiologic factor in this disease. Experimental infection with M. bovis in the absence of ultraviolet light produces a very mild disease (8). When daily doses of ultraviolet light are given, M. bovis infection will progress to severe keratoconjunctivitis. Exposure to ultraviolet radiation from sun lamps before and after experimental exposure to M. bovis can result in 100% of the eyes exposed developing IBK.

Direct contact with ocular and nasal discharge and mechanical vectors such as dust and the face fly, Musca autumnalis, are believed to be responsible for the spread of M. bovis within herds (4,30,32,40). Disease is most prevalent during times when face fly population and ultraviolet light concentrations are at their highest levels (11). M. bovis can survive for three days on the legs and feet of the fly, although it is destroyed in the digestive tract of the fly (40).

Laboratory reared face flies which are exposed to cultures of M. bovis can transmit the organisms to test calves. M. bovis can also be isolated from flies captured in herds having IBK by allowing the flies to walk on blood agar plates. There are no known natural reservoirs for M. bovis other than cattle (32).

Moraxella bovis will not establish itself in the eyes of rats, guinea pigs or rabbits (27). It can be established in the eyes of sheep, but will not produce disease. In mice it can also be established and will cause infection, but only after the mice are treated with ultraviolet light.

There is evidence that an infection with M. bovis can provide some immunity to future infections. In one study (10), cattle with no previous exposure to M. bovis were exposed to a known pathogenic strain of M. bovis. All of the eyes became infected, and 66% developed keratitis. After second and third reexposures, 55% became infected and only 3.4% developed keratitis. Those animals which did develop a keratitis had been reexposed to a heterologous strain of M. bovis.

Older cattle are more resistant to IBK than are cattle less than two years of age (32). In one five year study (11), the average infection rate for claves was 75%. For the adult animals it was 63%. Even more significant is that clinical signs of IBK were seen in 58% of the calves, but in only 16% of the adults.

The reasons for increased resistance of older cattle to infection are not known. Serum antibodies may or may not be important. When heterologous strains of M. bovis are used to challenge a vaccinated

animal, the amount of protection seen with homologous challenge is reduced or eliminated (34). The use of a formalin killed M. bovis vaccine, given intramuscularly, was ineffective in one study when the animals were allowed to live under natural environmental conditions (15). In a more recent study (16), calves vaccinated at birth with an autogenous formalin killed vaccine developed serum antibodies before they were extensively exposed to infection. The presence of these serum antibodies did not provide significant protection from the development of IBK. Weekly spraying of the eyes with Furazolidone was shown to be more effective than vaccination in reducing the incidence of clinical cases.

Different methods of vaccine preparation and vaccination procedures have been investigated. Heat killed vaccines are not effective (13). Formalin treated and live culture vaccines given intramuscularly are only 40% to 50% effective in preventing symptoms of IBK. These vaccines were even less effective in preventing establishment of M. bovis in the eye (13).

Pili vaccines have also been examined. In a study testing the effectiveness of a pili vaccine given intramuscularly, a greater number of the sera of resistant mice and cattle had antibodies to the pili antigen than those of vaccinated nonresistant animals (33). Pili vaccines prepared in oil and given into the dewlap proved more effective in stimulating specific serum antibodies and in lessening the severity and duration of the disease (35). Approximately 50% of the vaccinated calves still developed IBK after challenge. The use of

Mycobacterium paratuberculosis bacterin and oil in the pili vaccine does not increase its effectiveness (36). Disrupted cell vaccines are also ineffective (33). Vaccination into the third eyelid using a commercially prepared autogenous bacterin is reported as being only partially successful, not enough to be of commercial value (1).

The vaccination schedule is also important in determining a vaccine's effectiveness. In one study (14), it was found that at least 14 days should be allowed between successive doses of a formalin killed vaccine given intramuscularly, and a period of at least 42 days between the initiation of the vaccination and challenge with the organism. Animals should be vaccinated no later than the first week in May to have even partial protection by the beginning of the IBK season in mid or late June.

One of the first requirements for bacterial colonization is the attachment of the organism to the mucosal epithelium, and pili are thought in some cases to play a major role in this attachment. The presence of pili on Escherichia coli, isolated from urinary tract infections, is required for the organisms to attach to human uroepithelial cells in vitro (7). Strain K 12 E. coli and purified pili from this organism adhere very well to monkey kidney cells grown in tissue culture. The attachment appears to involve mannose containing receptors on the cell surface, since D-mannose can block the attachment (37). When nonpiliated or poorly piliated strains of E. coli, which do not attach to intestinal epithelial cells in vitro, are used to infect the small intestine of pigs, they become piliated. They also become

able to attach to epithelial cells in vitro (22). Fab antibody fragments specific for pili of related strains of E. coli are able to block adherence of unrelated strains (18). Thus epithelial cells apparently have different receptors for different pili. Purified pili of various strains of E. coli can competitively block attachment of the bacterial cells to procine small intestinal epithelial cells. The competition is mainly seen when homologous pili and bacteria are used.

Vaccination of pregnant gilts with purified pili provides protection to neonatal suckling pigs against diarrheal disease caused by enterotoxigenic E. coli strains having homologous pili (21). The vaccine does not protect against strains having heterologous pili.

Pili are also known to be a major factor in Neisseria gonorrhoeae infections. Virulence of N. gonorrhoeae is limited to colony types one and two which are piliated (19,41). Gonococcal attachment to amnion cells in tissue culture is facilitated by pili, as seen by both light and electron microscopy (42).

Purified pili and piliated gonococci can produce agglutination of human erythrocytes (5,6). By using antisera produced against the pili of different strains of gonococci, this hemagglutination can be blocked in some cases, but not in others. This suggests that the pili of N. gonorrhoeae also differ antigenically.

Freshly isolated cultures of M. bovis contain colonies that are flat, firm and dry and are described by some as "rough" (38) or "spreading-corroding" colony types (3). After continued subculture, these colonies become "smooth" or "nonspreading corroding" colony types.

The rough colonies autoagglutinate when suspended in physiological saline solution or distilled water, and the cells have many long pili on their surfaces (38). Cells from smooth colonies do not autoagglutinate and are devoid of pili.

M. bovis pili are peritrichously distributed over the cell surface, but tend to be more concentrated near the ends of the cell (39). They vary greatly in length, but are usually between 6.5 and 8.5 nm in diameter.

Rabbits immunized with viable whole cell preparations from rough colonies produce antibodies against pili (38). Rabbits immunized with viable whole cell preparations from smooth colonies do not produce antibodies against pili. This can be demonstrated by the development of precipitin lines in gel double-immunodiffusion tests where purified pili serve as the antigen. Antisera prepared against pili from one M. bovis isolate may or may not cross react with pili from different isolates. This suggests the existence of difference serotypes of M. bovis based on their pili antigens.

Only piliated forms of M. bovis are able to colonize the bovine conjunctival mucosa. Piliated hemolytic colonies used to infect cattle can be reisolated from 2 to 15 days later. Ultraviolet radiation of the eyes before infection enhances colonization. Hemolytic nonpiliated forms of M. bovis are cleared from the eye within one day after exposure, even if the animals are pretreated with ultraviolet light (24).

CHAPTER II

MATERIALS AND METHODS

Bacterial Cultures

The cultures used in this investigation were from three different sources. Strain 10900 was received from the American Type Culture Collection, Rockville, MD. Strain Fla 64 (6) was received from Dr. George Pugh, National Animal Disease Laboratory, Ames, Iowa. Strain 1646 was recently isolated from a herd of Angus cattle at The University of Tennessee.

All three strains were kept frozen at -70C until needed. For growth they were streaked onto 5% bovine blood agar prepared with freshly drawn citrated blood and Tryptose Blood Agar Base.[1] Cultures were incubated for 24 hours at 37 C.

All three cultures were hemolytic on bovine blood agar. Strain 1646 was piliated. Pili were not found on cells from strains Fla 64 (6) and 10900. All cultures were tested before each use and found to be gram negative diplobacilli, oxidase positive,[2] and gelatinase positive. The gelatinase test was performed on a medium identical to Bacto Staphylococcus Medium No. 110[3] except that it contained 5.0 grams of

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[1]Difco Laboratories, Detroit, Michigan.

[2]Oxidase Test Reagent Droppers, Marion Scientific Corporation, Kansas City, Missouri.

[3]Difco Laboratories, Detroit, Michigan.

sodium chloride rather than 75 grams per liter. They were also found to be negative for the reduction of nitrates,[4] negative for lactose and dextrose fermentation,[5] and negative for growth on Herellea and MacConkey Agar.[6] All strains produced the characteristic three banded reaction of M. bovis in litmus milk.[7] The presence or absence of pili was confirmed by negative staining.

Corneal Tissues

All corneal tissues were collected from freshly sacrificed animals. Bovine cornea were obtained at the Lays' Packing Company, Knoxville, Tennessee, within fifteen minutes after slaughter. Healthy appearing cornea were removed from the heads of animals of various breeds and ages. They were placed in sterile physiological saline or .01M TRIS buffer (pH 7.2) for transport to the laboratory.

Cornea from three young, mixed breed dogs were collected in similar manner at necropsy. These animals were being primarily used in a study involving tracheal tissues and the cornea appeared to be normal. They were placed in sterile physiologic saline for transport to the laboratory.

Procedures for Exposing Cornea to M. bovis

Exposure of piliated and nonpiliated strains. The first experiment involved piliated (1646) and nonpiliated (Fla 64 (6) and ATC 10900)

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[4] Bacto-Nitrate Agar, Difco Laboratories.

[5] Bacto-Kligler Iron Agar, Difco Laboratories.

[6] Difco Laboratories.

[7] Ibid.

strains of M. bovis. The organisms were grown at 37°C for 24 hours on 5% bovine blood agar. The growth was then scraped from the plate using a flamed inoculating loop and suspended in 10 ml of phosphate buffered saline (pH 7.2).

Cornea from four cattle were washed three times in PBS and cut in half with a single edged razor blade to produce four pieces of tissue from each animal. Each piece of corna from each animal was numbered 1 through 4 by punching holes along its periphery to denote which animal it was from. One section from each animal was then placed into one of four flasks containing turbid suspensions of either strain 1646, Fla 64 (6) of 10900. The fourth flask contained only PBS and served as the control. The flasks were then incubated for 15 minutes with gentle shaking in a 37°C water bath. The tissues were then removed from the bacterial suspensions, washed three times in PBS and placed in .1% glutaraldehyde in 0.1M sodium cacodylate buffer for 1 hour. They were then placed in .5% glutaraldehyde for 1 hour and then in 1% glutaraldehyde overnight at 4°C.

Cornea from the dogs and rabbits were treated in the same manner except only the piliated strain 1646 was used.

Exposure of heat and formalin treated M. bovis. Strain 1646 was grown overnight at 37°C on 5% bovine agar plates. The heat treated suspension was prepared by scraping the growth off one plate, suspending it in 2.0 ml of physiological saline and incubating it at 65°C for 1 hour. It was then kept at room temperature until the following day.

The formalin treated suspension was prepared by scraping the growth from one plate, suspending it in 1 ml of physiological saline and adding 1 ml of 5% formalin. This was incubated at room temperature until the following day.

Bovine corneal tissues were collected from four animals and prepared as before. Tissues from each animal were exposed to heat and formalin treated cell suspensions in the same manner described earlier. Tissues were also exposed to an untreated M. bovis suspension as a positive control and some were treated only with physiological saline as the negative control. Samples were incubated, washed and fixed in glutaraldehyde as described previously. All M. bovis suspensions were examined with negative staining for the presence of pili.

Competitive Inhibition by Purified Pili

M. bovis strain 1646 was used in this experiment as the source of purified pili and to measure attachment of the whole organism. Bovine cornea were collected as before from six animals. Each corneal tissue was cut into two sections, giving four tissue sections for each animal. One section from each animal was treated in one of the following ways. The competition assay was performed by first exposing the tissue to a purified pili suspension (0.8 mg of protein/ml). This was done by placing a section of tissue from each animal in a large plastic petri dish. The pili suspension in TRIS buffer (0.1M, pH 7.5) was added in drop wise fashion onto each tissue until it was covered. Fresh drops of the pili suspension were added every 10 minutes

for a total of 30 minutes. Tissues were kept at room temperature. After exposure to pili, they were washed three times in physiological saline. The tissues were then exposed to a turbid suspension of M. bovis in the same fashion. This suspension was prepared by scraping the growth of a 24 hour bovine blood agar plate and suspending it in physiological saline. The bacteria were also observed by negative staining to confirm the presence of pili. Additional drops of the cell suspension were added every 10 minutes for a total of 30 minutes. They were then washed three times in saline and placed in .25% glutaraldehyde for 1 hour. They were then placed in 1% glutaraldehyde for 1 additional hour and then into 2.5% glutaraldehyde at 4°C until the following day.

The pili treatment control was prepared by exposing tissue sections to pili as described above; however, instead of being exposed to M. bovis, drops of sterile saline were added for 30 minutes. The washings and fixation were the same as before.

The positive control for bacterial attachment was performed in the same manner as the competition assay except that TRIS buffer (.01M, pH 7.5) was added to the tissues instead of the pili suspension. All other procedures were identical.

The negative control was prepared by exposing tissue to only sterile saline and TRIS buffer. The incubations, washings and fixations were the same as used in the competition assay.

Pili Harvest and Purification

Moraxella bovis 1646 was chosen because it was known to be heavily piliated. The bottoms of eight steel pans were covered with Bacto G C medium base.[8] The pans were covered with aluminum foil and sterilized by autoclaving. They were then incubated overnight to test for sterility. A suspension of 1646 was swabbed over the agar surfaces and the pans were incubated for 24 hours at 37°C. The lawn of bacteria was scraped from the surface of the agar and placed in approximately 100 ml of TRIS buffer (.01M, pH 7.5) containing .01M sodium azide. The cells were then homogenized using a Virtis Homogenizer[9] at maximum speed for 20 minutes. The homogenizing flask was packed in ice. The homogenate was collected and centrifuged[10] for 1 hour at 3,000g and 1 hour at 15,000g. The supernatant was collected and centrifuged at 54,000g for 6 hours.[11] The pellet was suspended in approximately 30 ml of TRIS buffer and centrifuged for 1 hour at 100,000g. The pellet was resuspended in 10 ml of TRIS buffer, and the pili were precipitated by the addition of magnesium chloride to a final concentration of 0.1M. Following overnight incubation at 4°C, the precipitate was centrifuged at 15,000g for 1 hour and the pellet resuspended in 2 ml of TRIS buffer. This was then dialyzed

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[8]Difco Laboratories, Detroit, Michigan.

[9]Model 5-23, Virtis, Inc., Gardiner, New York.

[10]Sorvall RC2-13, Ivan Sorval, Inc., Newtown, Connecticut.

[11]Sorvall OTD-65 Ultracentrifuge, Dupont Instruments, Newtown, Connecticut.

for approximately 48 hours at 4°C against frequent changes of TRIS buffer. The pili were then frozen at -40°C until used. Protein content was determined to be 0.8 mg/ml by the Lowry method.

Scanning Electron Microscopy

Specimens were prepared for scanning electron microscopy by fixation with glutaraldehyde as previously described. The bovine tissues were dehydrated by passage through solutions of 10, 20, 30, 40, 50, 60, 70, 80, 90, 95, and 100% acetone for 20 minutes each. The dog and rabbit cornea were kept in each acetone dilution for only 10 minutes because they were thinner and dehydrated faster. The specimens were critical point dried using a Bomar SPC-900/EX Critical Point Dryer.[12] After trimming and mounting on stubs, the specimens were coated with gold in a vacuum evaporator.[13]

Attached cells were counted in the following manner. The ETEC Autoscan Scanning Electron Microscope[14] is equipped with a 7.25 x 7.25CM viewing screen which has an area of 52.5 cm^2. By counting the bacteria seen on the screen, and dividing by the actual surface area observed, it is possible to calculate the number of attached bacteria/cm^2 of tissue. The electron microscope is also equipped with calibrated controls for moving the specimen 25mm along x and y coordinates. By moving the specimen .5mm for five times along

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[12]Bomar, Tacoma, Washington.

[13]Denton Vacuum DV-515 Automatic Evaporator, Denton Vacuum, Inc., Cherry Hill, New Jersey.

[14]ETEC Corporation, Hayward, California.

the x coordinate and five times down the y coordinate, a total of 25 squares were counted giving a total of .438 cm^2 with the magnification set at 1,500. This magnification setting is for the photographic screen and must be multiplied by two to equal the area of the viewing screen. Each tissue sample was counted in this manner to eliminate bias.

Transmission Electron Microscopy

Negative staining was used to verify the presence or absence of pili on the M. bovis strains used. This technique was also used to examine the pili preparation. One-hundred mesh copper grids[15] were coated with a collodion film[16] which was then carbon coated and glow discharged with the Denton Vacuum Evaporator. A suspension of M. bovis in distilled water, or pili in TRIS buffer, was placed on the grids for approximately one minute. The drops were then gently removed by tapping the grid, and drops of .7% phosphotungstic acid stain (pH 7.0), were added. Stain was usually applied and removed immediately three times. The grids were allowed to dry and were examined with a Zeiss Electron Microscope.[17] The incubations, washings and fixations were the same as used in the competition assay.

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[15]Ernest Fullam, Inc., Schenectady, N.Y.

[16]Lodd Research Industries, Burlington, Vermont.

[17]Model EM 95, Carl Zeiss, Inc., New York, N.Y., and TRIS buffer.

CHAPTER III

RESULTS

Exposure of Piliated and Nonpiliated Strains

Strain 1646 was found to be highly piliated, and strains 10900 and Fla 64 (6) were nonpiliated as seen in Figures 1 and 2. Strain 1646 adhered to bovine cornea in much greater numbers than did the nonpiliated strains, as seen in Figures 3 and 4. The numbers of bacterial cells of each strain adhering to bovine cornea are found in Table I. Few nonpiliated cells were found on the corneal surface and the control tissues had no adhering bacteria of any form. When 1646 was exposed to rabbit and dog cornea, the results were identical to those of nonpiliated cells attaching to bovine cornea. Less than ten cells were counted on each specimen of rabbit and dog cornea.

Exposure of Heat and Formalin Treated M. bovis

Heat and formalin pretreatment of M. bovis 1646 produced the results shown in Table II. Although there was considerable variability from one animal to another, by using the test for paired comparisons (17), a significant difference was found between the attachment of heat treated and formalin treated cells at the 5% confidence level. Only one control using cornea and untreated M. bovis was done; the number of attached bacteria was similar to those of cornea exposed to formalin pretreated cells. Negative staining of the bacteria revealed that heat treatment severely affected the pili by either destroying



Figure 1. Moraxella bovis, strain 1646, notice many long pili. PTA negative stained. X 16,000

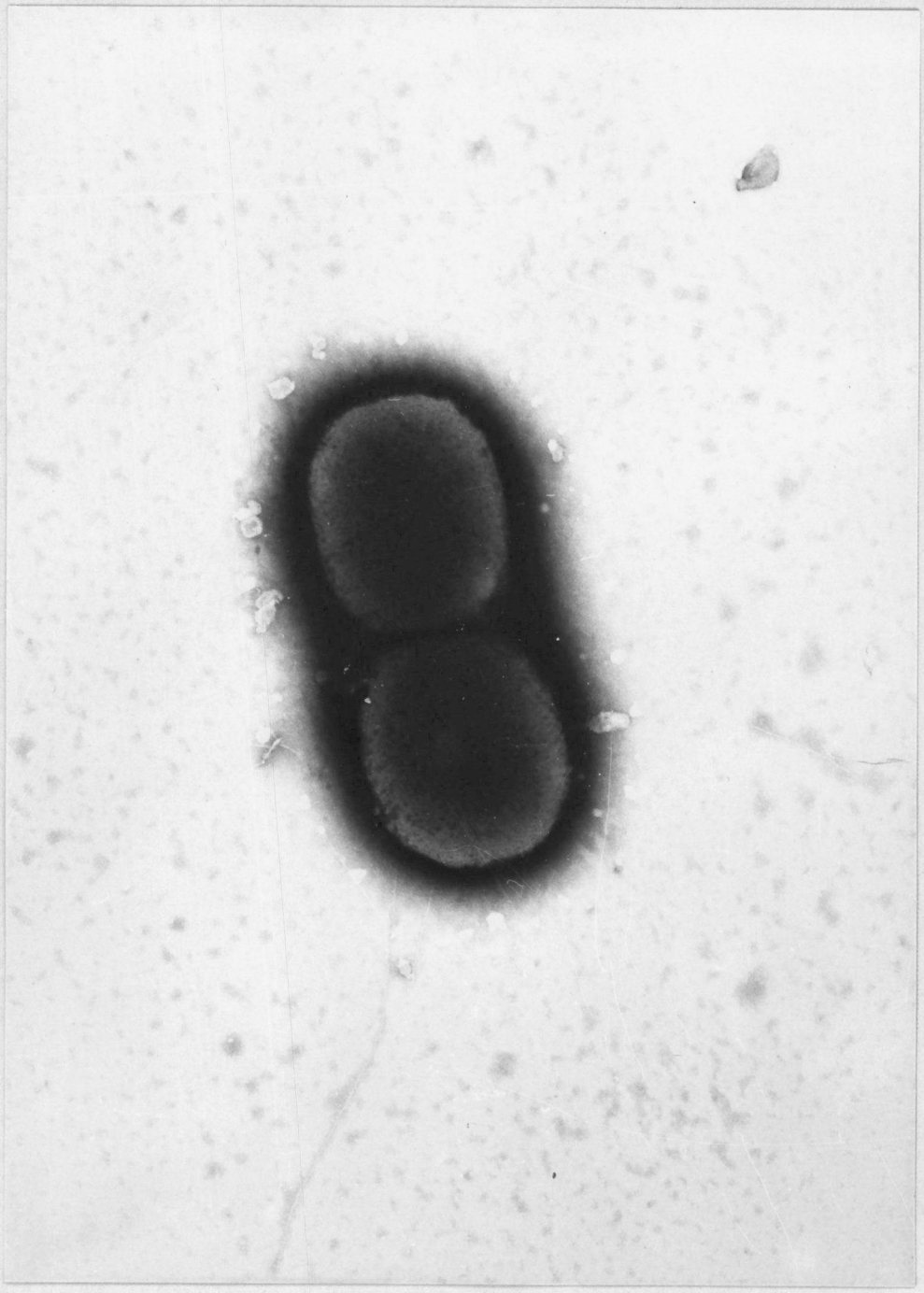

Figure 2. Moraxella bovis, strain 10900. Only rare cells were observed with pili. Pili, when present, were greatly reduced in numbers. PTA negative stained. X 20,000

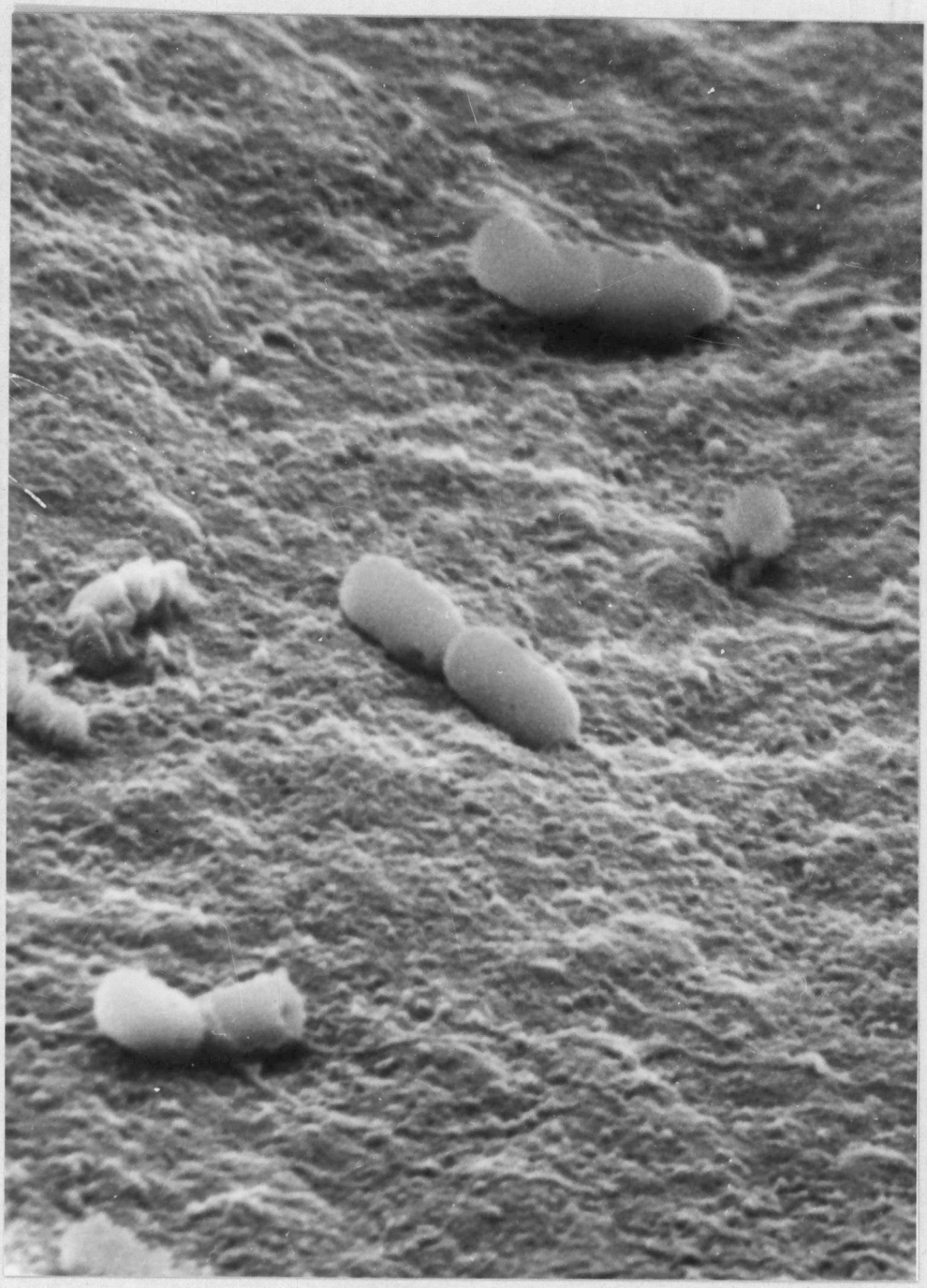

Figure 3. Moraxella bovis, strain 1646, attached to bovine cornea. SEM X 6,600

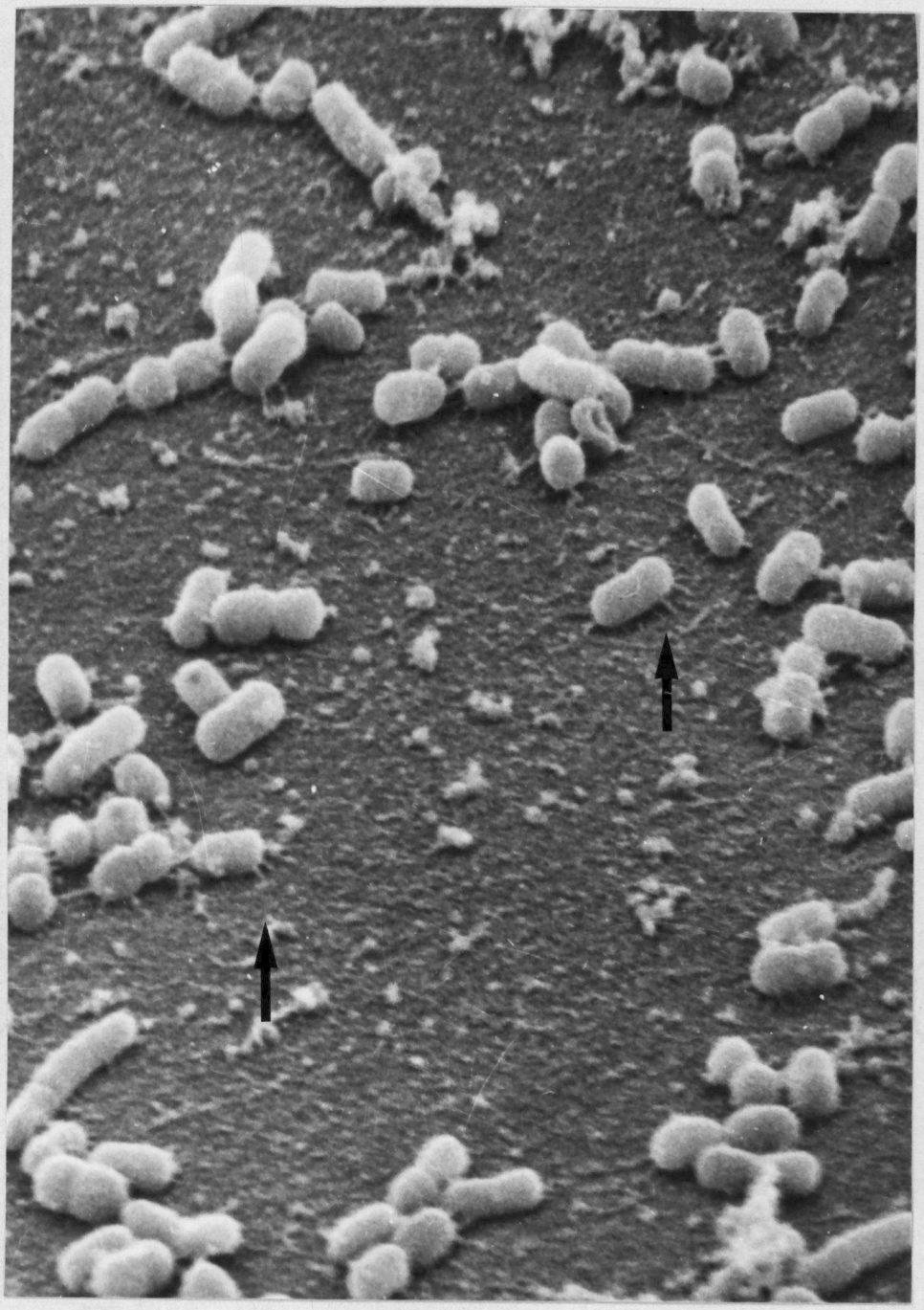

Figure 4. Moraxella bovis, strain 1646, attached to bovine cornea with filamentous protrusions from the bacterial cell. These filaments may represent bundles of pili. SEM X 4,000

TABLE I

ATTACHMENT OF PILIATED AND NONPILIATED STRAINS OF *MORAXELLA BOVIS* TO BOVINE CORNEA

Strain of *M. bovis*	Cornea Number[a]				Average number of attached bacteria/cm^2 ± S. D.
	1	2	3	4	
1646	228[b]	142	331	297	249.5 ± 83.5
10900	<10[c]	<10	<10	<10	----------
Fla 64 (6)	<10	<10	<10	<10	----------
neg. control	0	0	0	0	0

[a]Each corneal specimen was obtained from a different animal.

[b]Attachment expressed as number of cells/cm^2.

[c]<10 organisms were seen when the entire surface of the tissue was scanned, which represents an area greater than that used to count tissues which had attached bateria.

TABLE II

ATTACHMENT OF PILIATED *MORAXELLA BOVIS* TO BOVINE CORNEA AFTER PRETREATMENT OF THE BACTERIA WITH HEAT OR FORMALIN

Treatment	Cornea Number[a] 1	2	3	4	Average number of attached bacteria/cm^2 ± S. D.
65 C	311[b]	59	224	*	198 ± 128
5% formalin	728	1130	1010	*	956 ± 206
no pretreatment	1050	ND	ND	ND	1050
no *M. bovis*	0	ND	ND	ND	0

[a]Each corneal specimen was obtained from a different animal.

[b]Attachment expressed as number of cells/cm^2.

*The cornea from animal number 4 had large areas of surface epithelium missing and was not counted.

their shape and size, as seen in Figure 5. The cells which were pretreated with formalin had many long, normal appearing pili.

Competitive Inhibition by Purified Pili

The results for the competitive blocking of M. bovis 1646 using homologous purified pili (Figure 6) are shown in Table III. At the 5% confidence leve, the pretreatment of bovine cornea with purified pili was found to significantly reduce the number of bacteria attaching to the tissue. No bacteria were seen in controls which received either no treatment or pili only. Pili treatment alone did not alter the appearance of the cornea.



Figure 5. Moraxella bovis, strain 1646, after heat treatment, note damaged pili. PTA negative stained. X 26,600

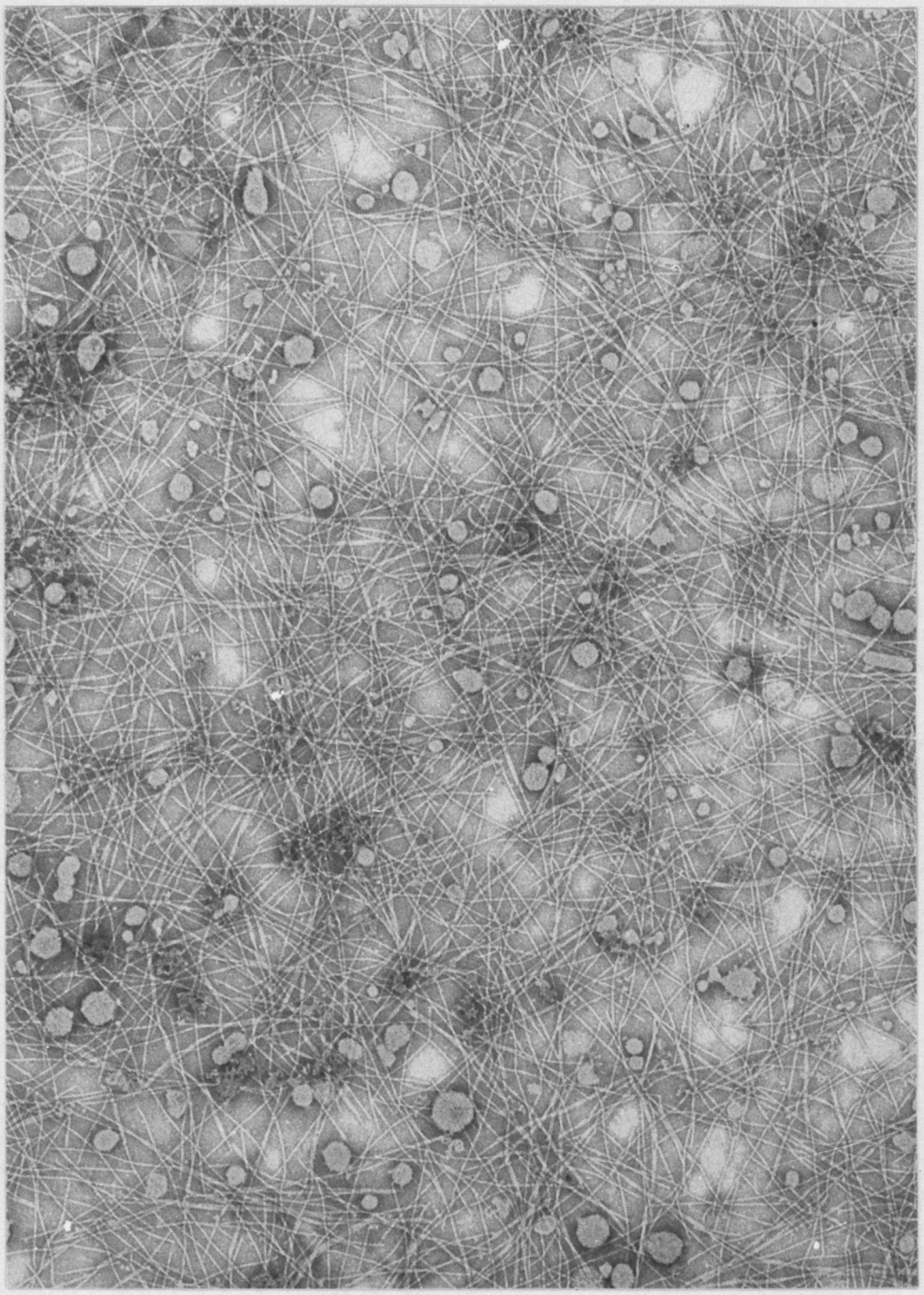

Figure 6. Purified pili preparation from Moraxella bovis, strain 1646, showing pili and some remaining cell debris. PTA negative stained. X 40,000

TABLE III

ATTACHMENT OF PILIATED MORAXELLA BOVIS TO BOVINE CORNEA PRETREATED WITH HOMOLOGOUS PURIFIED PILI

Treatment	Cornea number[a] 1	2	3	4*	5	6	Average number of attached bacteria/cm^2 ± S. D.
pili + M. bovis	144[b]	41	146	59	9	155	92 ± 63
M. bovis only	246	183	342	247	260	210	248 ± 54
pili only	0	ND	ND	ND	ND	ND	
no treatment	0	ND	ND	ND	ND	ND	

[a]Each corneal specimen was obtained from a different animal.

[b]Attachment expressed as number of cells/cm^2.

CHAPTER IV

DISCUSSION

Moraxella bovis is known to cause the severe infection of the eyes of cattle known as infectious bovine keratoconjunctivitis. Piliated and nonpiliated forms of the organism are known to exist. Upon isolation from an animal having IBK, the organism usually forms "rough" colony types which are characteristic of the piliated form of the bacteria.

Previous work (24) has shown that only the piliated form of M. bovis can colonize the bovine conjunctival mucosa. Nonpiliated bacteria are cleared from the eyes within one day of instillation. A study on the effectiveness of pili vaccines has shown that a greater number of resistance mice and cattle have antibodies to the pili antigen than do vaccinated nonresistant animals (33). These facts suggest that the pili of M. bovis are a major contributing factor in the development of IBK. The studies reported in this thesis add supporting evidence to this fact.

The use of freshly collected whole corneal tissues, and the techniques of scanning electron microscopy, allowed for the attachment of bacteria to be observed in detail under conditions approaching those found in nature. The tissues used in these experiments were in the glutaraldehyde fixative within two or three hours after removal from the donor, and were in excellent condition.

The first experiment comparing the attachment of piliated and nonpiliated strains of M. bovis demonstrated the superior ability of the piliated cells to attach to corneal tissue. It is important to remember, however, that the presence of pili on M. bovis is not a stable condition. Since completion of these experiments, nonpiliated forms of strain 1646 have been seen after repeated subculture. The strains ATCC 10900 and Fla 64 (6) may well have been piliated at some time before they were received, or they could have lost their pili after they had been subcultured.

Moraxella bovis is known to autoagglutinate in physiological saline solutions and in phosphate buffered saline. This was found to be true in the suspensions of the piliated strain 1646 used to measure adherence; however, if the suspension was shaken continuously, not all of the cells agglutinated. Therefore, .85% saline was used to expose the cornea to the bacteria. M. bovis was found to suspend well in distilled water with no harmful effects to the cells or their pili. In a recent experiment, cells suspended in distilled water were shown to be fully infectious.

M. bovis cannot be established in the eyes of rats, guinea pigs and rabbits (27), nor has it been reported to be carried by any other animal under natural conditions. Pugh has reported an organism similar to M. bovis in horses, except that it is not infectious for cattle (12). The inability of the organism to adhere to dog and rabbit cornea, as shown in these experiments, suggests that the reason it does not naturally occur in other animal species is because it cannot attach to other corneal surfaces.

Killed-cell vaccines prepared in earlier studies by heating the organism proved to be completely ineffective in providing protection against IBK (13): whereas formalin killed vaccines have shown to produce some protection (13). Negative staining has shown that heat treatment damages the pili whereas formalin treatment does not. Significant reduction in attachment of heat treated cells was observed. The formalin treated cells retained their ability to attach to bovine cornea. All of these facts suggest that pili are important not only in the development of IBK, but in the development of immunity to the disease as well.

Additional evidence that pili are responsible for attachment of M. bovis to bovine cornea was provided by the competitive blocking of attachment through the pretreatment of the cornea with purified pili. There was a significant reduction in attachment of piliated cells to the pretreated cornea. These results are similar to those reported with E. coli which was shown to be competitively blocked from attaching to porcine small intestinal epithelial cells by homologous pili (18).

The importance of pili in the attachment of M. bovis to bovine cornea has been more clearly established. These findings indicate that the blockage of attachment, possibly through vaccination with a purified pilus vaccine, could be the most effective method of controlling IBK.

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VITA

Wallace Greene was born in Sumpter, South Carolina, on November 22, 1954. He attended elementary schools in Knoxville, Tennessee, and was graduated from West High School in June 1972. He then entered The University of Tennessee, and in March 1976 he received a Bachelor of Arts degree with a major in Microbiology. In the spring of 1976 he was accepted into the graduate program at The University of Tennessee and received a teaching assistantship. He received a Master of Science degree in Microbiology in August 1979.

The author is a member of Pi Kappa Alpha and the American Society for Microbiology. Mr. Greene is presently employed at The University of Tennessee College of Veterinary Medicine where he is continuing to study IBK.

He is married to the former Jenny Downen of Knoxville, Tennessee.