Comparison Of Three Strains Of Contagious Equine Metritis Organism By Polyacrylamide Gel Electrophoresis And Description Of Three Colonial Opacity Variants

Albert Olszewski

Carroll College, Helena, MT

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COMPARISON OF THREE STRAINS OF CONTAGIOUS EQUINE METRITIS ORGANISM BY POLYACRYLAMIDE GEL ELECTROPHORESIS AND DESCRIPTION OF THREE COLONIAL OPACITY VARIANTS

Submitted in partial fulfillment of the requirements for graduation with honors to the Department of Biology at Carroll College, Helena, Montana.

Albert David Olszewski
April 2, 1984
This thesis for honors recognition has been approved for the Department of Biology.

Dr. James J. Manion, Advisor

Fr. Joseph Harrington

Dr. Barry Terst

April 2, 1984
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ABSTRACT

The colonial morphology and molecular composition of 26 phenotypes of Contagious Equine Metritis Organisms (CEMO) were studied by stereomicroscopic examination and polyacrylamide gel electrophoresis. Twenty-three phenotypes had colonies that were round and smooth. Three phenotypes had colonies that were round and smooth with occasional fimbria projecting from the edge of the colonies. The consistency of all the colonies varied from friable and butyrous to viscid. Based on external observation in reflected and transmitted light, the 26 phenotypes were separated into 3 different types of colonial variants based on opacity. The major stained bands in the page profiles of all 26 phenotypes were observed and recorded. The stained bands were observed in polyacrylamide gels that were prepared with CBB stain, silver stain, $^{125}$I-surface labeling, and Immuno-electroblotting procedures. Several differences between the polyacrylamide gel profiles of these strains were observed.
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INTRODUCTION

Contagious Equine Metritis (CEM) is a venereal disease of horses. It first appeared in 1977 in the thoroughbred population in the United Kingdom (7). This disease causes acute endometritis and temporary infertility in the mare. The stallion is asymptomatic. At the present time this disease has been reported on 5 continents (13)(42)(29)(25)(10)(39). CEM can be diagnosed by isolating the causative agent from the genital tract of mares, and the external genitalia of stallions. Treatment involves the application of a variety of antibiotics and is successful in all stallions and most mares. A small proportion of the affected mare population have remained chronic carriers after treatment.

The causative agent of CEM is a small gram-negative coccobacillus that is unofficially named Contagious Equine Metritis Organism (CEMO). In 1978, Taylor et al. (45) proposed that this organism be accepted as a new species of *Haemophilus*, named *Haemophilus equigenitalis*. However, the proposal was not accepted by the International Committee on Systemic Bacteriology. CEMO is a non-motile, fastidious, asaccharolytic, non-proteolytic organism. It is cultivated in a moist atmosphere with an increased carbon dioxide content at a temperature of 37°C. Also, this organism has catalase, cytochrome oxidase, leucine aminopeptidase, esterase, and acid and phosphatase activity. It is nonreactive to all other biochemical tests.
In 1981, Sahu et al. (37) reported the isolation of 5 morphological types of CEMO bacterial colonies from uterine and cervical samples of CEM-infected pony mares. Besides size, shape, and general appearance, nothing else was described in the results, and very little was discussed pertaining to colony morphology.

The purpose of this investigation is to compare three strains of CEMO by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and to compare 26 colonial variants of CEMO from these strains. This thesis reports several differences between the polyacrylamide gel profiles of these strains and 3 colonial variants based on opacity.
LITERATURE REVIEW

I. History

Cases of a previously undescribed equine genital infection were first reported in the British Isles (7)(46) during the spring of 1977. The first cases to be reported occurred on a large stud farm in the Newmarket, England, area. Affected mares had a copious vaginal discharge, and returned to estrus after a shortened diestrus period. The disease appeared to be transmitted sexually although the stallions were asymptomatic. During 1977 breeding season, this equine genital infection was reported on other stud farms in the Newmarket area. Approximately 200 mares, and 23 stallions were infected by the end of the 1977 breeding season (31).

After extensive laboratory studies, a gram-negative coccobacillus was isolated and cultured under conditions of increased CO₂ tension from equine cervical and vaginal pus. This organism was shown to be the causal agent of the genital infection based on the evidence produced by transmission experiments (26) and field observations (46)(32).

A preliminary investigation on the properties of the organism documented that the organism was non-motile, positive to the oxidase, catalase, and phosphatase tests, but non-reactive to all other biochemical tests. Antimicrobial sensitivity tests demonstrated that the organism was sensitive to a large number of antibiotics, including benzyl penicillin,
ampicillin, and chlorhexidine, but was highly resistant to streptomycin (31). Attempts to establish identity with previously characterized bacteria were unsuccessful and the organism was considered to be a heretofore unidentified pathogen.

The consequences of this genital infection on the thoroughbred industry were serious. In 1977, CEMO was officially confirmed in Britain, Ireland, and Australia (8)(46). Stud farms with reported cases of CEM had to curtail their breeding programs. On some stud farms, fertility was reduced by 40 percent (13). In the fall of 1977, many countries including the United States, and Canada, imposed a ban on the importation of horses which might carry the disease from United Kingdom, Ireland, and France.

In 1978, CEM was reported in the thoroughbred population of Kentucky (42), France (25), Germany (24), and Belgium (29). From the Kentucky outbreak, both streptomycin sensitive and resistant strains were isolated. In 1979, there was a CEM outbreak in Missouri (16), and by the end of 1980 the disease was confirmed in Japan (39) and Brazil (10). CEM has been diagnosed mainly in thoroughbreds, but it has also been identified in other breeds of horses and one breed of pony (46).

II. Characterization of the Organism

CEMO is usually described as a small non-motile gram-negative coccobacillus, but may be observed as monomorphic short rods. Pleomorphism has been reported among organisms that have been incubated for extended periods of time (37). Under the
electron microscope, the bacterium exhibits the typical structure of gram-negative cell walls and is enveloped in a very thin capsule that can only be demonstrated when the bacterium is stained with Ruthenium red following preparation of ultra-thin sections (40). No pili or flagella have been observed on the CEMO.

Taylor et al. describes CEMO as a fastidious organism that grows best on heated blood (chocolate) agar in an atmosphere of 5 to 10 percent carbon dioxide. Growth is stimulated by, but does not require, hematin factor X, factor V, or factor XV. The condition that will produce superior growth is incubation in a McIntosh and Fieldes jar evacuated to 570 mm mercury and refilled with 90 percent hydrogen and 10 percent carbon dioxide with the palladium catalyst removed. The optimal temperature for growth is 37°C, but CEMO growth has been reported over a temperature range of 30°C to 40°C. Growth does not occur or is very poor at 22°C (45).

Tainturier et al. characterized CEMO as non-fermentative, non-proteolytic bacterium that has catalase, cytochrome oxidase, leucine amniopeptidase, esterase, acid and alkaline phosphatase activities using both conventional methods and API ZYM (API Systems SA.A, La Balme les Grottes, Montalieu Vercieu, France) system of enzyme detection (44). The organism has a DNA composition of 36.1 percent GC (47). CEMO appears to metabolize independent of glycolysis and the hexose monophosphate pathway, and is dependent on the TCA cycle and
oxidative phosphorylation for cell energy (23).

CEMO is sensitive to a number of drugs. All strains are susceptible to a wide range of drugs including beta-lactam antibiotics, chloramphenicol, nalidixic acid, and nitrofurantoin. All but two strains are highly resistant to streptomycin. All strains of CEMO are resistant to clindamycin, linomycin, and metranidazole (10).

III. Pathogenesis of the Disease

CEM is highly contagious and is primarily spread by venereal transmission. Ineffective antiseptic conditions have contributed to the spread of the disease. Vertical transmission of CEM may occur by congenital antepartum infection, and parturient contamination of foals from positive mares (30)(38).

Both the mare and the stallion have been recognized as carriers of CEMO. The organism has been isolated from the clitoral sinus, cervix, and uterus of the mare for periods up to 24 months after natural and experimental infections (43) (27). Also, this organism has been cultured from the vagina, oviducts, and ovaries of some mares (1). In the stallion the CEMO is isolated from the skin and mucosal surfaces of the external genitalia. The organism persisted in these locations on an experimentally infected pony stallion for 196 days (28), and at least 180 days in two stallions that had been imported from France to Kentucky in 1977 (6).

IV. Clinical Signs

Contagious Equine Metritis is characterized by the inflammation of the endometrium, vagina, and cervix. Infertility can
be caused by a CEMO Infection (11). In acute cases a copious vaginal discharge is observed. The discharge varies from a frank pus to a sticky grey mucopurulent fluid that is observed on the lips of the vulva. Other signs are the "matting" of the tail and fluid running down the hindquarters of the mare. In less severe cases, no discharge is seen but a pool of fluid may accumulate on the floor of the vagina. Examinations of the genital tract have revealed severe vaginitis, and cervicitis with pus discharging from the cervix. Redness of the vagina and cervix, produced by extreme hyperemia, is more noticeable than in other types of genital infections (31). Symptoms are usually seen 2 to 10 days after copulation, but in a few instances they have not appeared until 80 days (32). Clinical signs may persist for a variable time ranging from days to weeks. CEMO can still be recovered intermittently for a considerable period of time after the clinical signs disappear (47). The affected mares generally return to estrus within 8 to 15 days and show no sign of systemic infection.

All foals born to mares that contracted CEM have been reported healthy. In one case, CEMO was isolated from the penile sheath of a colt up to 3 months of age (29). Clinical signs have not been observed in stallions (15).

The severity of these symptoms likely depends on several factors. These include the dose of inoculum, virulence of the organism, and the immune status of the host. Mares experimentally infected have shown that the virulence of organisms subcultured
in vitro was inversely proportionate to the number of times the organisms had been subcultured in the laboratory. Also, the clinical response of ponies reinfected was less severe than in ponies receiving a primary infection (17)(37).

V. Pathology

The opportunity to study the pathological and histological changes caused by CEMO has been limited because it is not a fatal disease. Thus, pathological investigations have been restricted to a small number of pony and mixed breed mares that have been experimentally infected, killed, and examined in studies to observe the pathogenesis of the disease (28)(2), and to the histological study of endometrial biopsies obtained from field and experimental cases (33)(34).

The pathological changes appear to be confined to the genital tract, where the endometrium is the principal site of change. In acute stages of the disease there is a severe diffuse endometritis and cervicitis that becomes more severe subacute and predominantly plasmacytic within 14 days after copulation and then declines (3). Vaginitis reaches its peak of moderate severity by 14 days and then declines until it disappears. Inflammation of the oviducts is observed consistently near the ampulla where the mucosa is thick and highly folded. Also, inflammation is seen intermittently near the isthmus where the mucosa is thinner and is not highly folded (2).

The histopathological changes of the endometrium seen in CEM are not totally different from those of the more common
bacterial infections of the genital tract (2). In the superficial layer, stratum compactum, and stratum spongiosum, severe edema is observed as early as 2 days after the inoculation. Severe edema is evident up to 11 days but is mild by 14 days. Diffuse cellular infiltration of the superficial layer, stratum compactum, and stratum compactum, and stratum spongiosum occurs throughout the infection. The concentration of neutrophils, lymphocytes, macrophages, and plasma cells varies in each endometrial layer as CEM progresses. Erosion or hyperplasia of the luminal, ductular, or glandular epithelium was not observed at any stage of CEM. The histopathological changes of the uterus and cervix are the same as those of the endometrium but not as severe (2).

VI. Diagnosis

CEM is confirmed by the isolation of the gram-negative coccobacillus from the genital tract of a mare or stallion. In order to reduce the possibility of obtaining a false negative result careful procedures for obtaining samples must be strictly adhered to (31).

The appropriate sites for obtaining samples from a mare are the endometrium during early estrus, and from the clitoris and its sinuses. The advantages of swabbing the clitoris and its sinuses are that the sampling may be performed on a baren mare at anytime, and on an infoal mare at any stage of the pregnancy. The study by Timoney et al. (1981) emphasizes taking swabs from the appropriate locations on more than one occasion to increase the effectiveness of detecting a carrier mare (48).
To obtain satisfactory samples from the stallion, swabs should be taken on at least three occasions from the fossa glandis and the folds of the penile sheath. A selective media with streptomycin must be used to isolate CEMO from a stallion in order to prevent contamination of the culture plates by unwanted bacteria from the external surfaces of the genital tract. This has made it difficult to isolate the streptomycin-sensitive strains of CEM from stallions (31).

Careful attention should be paid to hygiene during collection. Disposable gloves should be worn and changed between examinations. Uncontaminated samples should be guarded against contamination by placing swabs in Amel's charcoal medium at 4°C for delivery to the laboratory (15).

Swabs from the equine genital tracts should be inoculated onto blood and chocolate agar plates. Blood agar plates should be incubated aerobically for two days at 37°C, and the chocolate agar plates in an atmosphere of 5 to 10 percent carbon dioxide at 37°C for at least 6 days. Within 48 to 72 hours, suspect colonies may be observed on the chocolate agar, but not on the blood agar (31).

Laboratories in Europe and the United States have developed serological techniques for diagnostic purposes. Benson and co-workers developed a serum agglutination tube (SAT), antiglobulin test, and a complement fixation test (CF) (4)(9). They suggest a titer of 1/80 be considered evidence of recent
CEM infections. A peak SAT and CF antibody response is seen between 14 and 21 days with the CF antibody developing later than SAT antibody, but persisting for a longer period of time (12). The CF test is valuable in confirming recent cases of CEM infection in mares. A complement fixation test developed by Bryans and co-workers was successfully used as a diagnostic aid during the Kentucky outbreak of 1978 and is now used as an official routine test to control the disease (5). A passive hemagglutination test has been developed that is not affected by substances that could inhibit the complementary system. This test is considered to be a very sensitive test (18).

These various serological tests have proved to be valuable complements to bacteriological isolation in identifying infected mares during an acute outbreak of CEM. It seems that mares with endometritis caused by a CEM infection develop a humoral response that can be detected for up to 40 days after inoculation. However, these tests appear to have little value in identifying chronically infected or carrier mares because either antibody levels have become very low or the bacteria has localized itself in the reproductive tract and does not stimulate a humoral response (31).

VII. Treatment and Control

The majority of all mares infected with CEM will recover spontaneously without treatment. CEMO can no longer be isolated from these mares. A large number of the mares that do not recover spontaneously will recover after treatment. A small proportion
of the mares will remain carriers despite treatment and have proved to be the source of fresh outbreaks of CEMO.

Infected mares have been treated by a wide variety of antimicrobial agents given by topical and parental route. Uterine infusions of benzyl penicillin, ampicillin, neomycin, introfurazone, and chlorhexidine in combination or alone are given daily for 5 to 7 days. This accompanied by parenteral treatment with penicillin or ampicillin given daily for a similar period. The clitoris and sinuses are completely cleansed with a 4 percent solution of chlorhexidine. Then the clitoris is dressed with a nitrofurazone ointment. This procedure is repeated at 24 to 48 hour intervals on 5 occasions (31).

The treatment of a stallion consists of thorough and careful washing of the erect penis, fossa glandis, and penile sheath with a solution not less than 2% chlorhexidine and then dressing with an ointment of not less than 0.2% nitrofurazone. Treatment is repeated daily for 5 to 7 days. Further treatment is necessary if CEMO is cultured from the treated stallion at a later time (15).

At this time, the most effective controls to prevent outbreaks of CEM are the extensive serological and bacteriological screening techniques that can detect and diagnose CEM clinical and carrier cases, the implementation of high standards of hygiene on stud farms, and the cessation of breeding "high risk" mares. Other methods of control include introducing codes of practice, placing bans on the importation of horses
from countries with confirmed cases of CEM, and surgical removal of the clitoris or the clitoral sinuses from "high risk" mares. Vaccination against CEM has been tried, and has not been successful (17)(36). Artificial insemination is considered the best method of preventing the spread of the disease but it is currently not an acceptable procedure for the registration of thoroughbred foals (35).
METHOD AND MATERIALS

Bacteria and culture conditions. The three strains of CEMO used were supplied by P.J. Hitchcock (Laboratory of Microbial Structure and Function, Rocky Mountain Laboratories, National Institutes of Health, Hamilton, MT) who originally obtained them from T.W. Swerczek (Department of Veterinary Science, University of Kentucky, Lexington, KY). Two strains of CEMO used, 289 (streptomycin sensitive), and 188 (streptomycin resistant) are isolates from Kentucky. The third strain of CEMO used, 48 (streptomycin sensitive) is an isolate from Germany. The organisms were grown on CEMO clear typing medium (19) which is a modification of the medium described by James (20). The following was added to each liter of medium: 3.75 g of trypticase peptone (Baltimore Biological Laboratories BBL, Cockeysville, MD); 7.5 g meat peptone # (BBL); 4 g K$_2$HPO$_4$ (crystalline); 5 g NaCl; 2 g soluable starch (BBL); and 11 g Bacto agar (Difco Laboratories, Detroit, MI). The medium was autoclaved at 20 lb/in$^2$ for 15 minutes. Ten ml of Isovital X (BBL) was thoroughly mixed into the cooled (56°C) medium before pouring plates. Two milliliters containing hemin (1 mg/ml) (Sigma Chemical Co., St. Louis, MO), L-histidine (1 mg/ml), and NAD (0.3 mg/ml) (Sigma) were added. Medium for growth of streptomycin sensitive organisms required two additional components: 2 ml each of serine (0.05 g/ml), and alanine (0.1 g/ml) were added to the cooled medium before pouring. The plates were stored at 4°C. Cultures were incubated in a
moist atmosphere containing 5% carbon dioxide in air at 37°C for 72 to 96 hours.

Colony morphology, light microscopy, and photography. The colony morphology of 96 hour CEMO cultures was studied with a stereomicroscope (Stereo Zoom 7, Bausch and Lomb, Rochester, NY) that was equipped with a substage reflecting mirror with diffrusing (reflecting light) and plane polished (transmitted light) surfaces. Microphotography of the colonies was accomplished with a Canon F+b camera attached to the stereomicroscope. The film used to photograph the colonies was Plus - X film (Kodak Co., Rochester, NY).

SDS-PAGE. Preparations of CEMO were subjected to SDS-PAGE using the Laemmeli buffer system (22). The bis to acrylamide ratio was 0.8 g to 30 g. The 4 percent stacking gel, and the 12.5 percent separating gel did not contain SDS (M. Wycoff, R. Rubbard, A. Chrambach, Fed. Proc. 35: 1383, 1976). Electrophoresis was accomplished at 40 mA constant current using Tris-glycine (pH 8.3) with 0.1 percent SDS buffer. Molecular weight markers from Bio-Rad laboratories (Richmond, CA) were used in all gels made. The molecular weight markers were phosphorylase (94k daltons [Kd]), bovine serum albumin (68 Kd), ovalbumin (43 Kd), carbonic anhydrase (30 Kd), soybean trypsin inhibitor (21 Kd), and lysozyme (14.3 Kd).

CBB stain. Gels were fixed overnight in 25 percent (vol/vol) isopropyl alcohol in 7 percent (vol/vol) acetic acid. Fixed
gels were stained in 2 percent CBB R250 (Fisher Scientific Co., Fairlawn, NJ) in 25 percent (vol/vol) isopropyl alcohol in 7 percent (vol/vol) acetic acid.

Whole cell (WC) lysates and Proteinase K (PK) digestion. WC lysates and PK digests were made following the procedure described by Hitchcock (19). Organisms, grown for 72 hours, were carefully scraped off agar plates with sterile dacron swabs and suspended in Dulbecca's phosphate-buffered saline (14) to a turbidity of 100 klett units (Klett-Summerson colorimeter, blue filter, 12.5 mm path length). 1.5 ml of this suspension was placed in microfuge tubes and centrifuged in a Microfuge B (Beckman Instruments Inc., Fullerton, CA) for 2 minutes. Pellets were resuspended in 50 microliters of 2 percent SDS lysing buffer containing 1 percent SDS (British Drug House, Poole, England), 4 percent 2-mercaptoethanol, 10 percent glycerol, 1 m Tris (pH 6.8), and bromphenol blue. This suspension was heated at 100°C for 10 minutes with vortexing. For protein digestion, 25 micrograms of proteinase K (PK) (Boehringer Mannheim GmbH, West Germany) solubilized in 10 microliters of lysing buffer was added to each boiled lysate and incubated at 60°C for 1 hour. WC lysates and PK digests were loaded into 12.5 percent polyacrylamide gels in either 5 or 10 microliter aliquots.

Silver stain. The silver-lipopolysaccharide and protein staining procedure was used as described by Hitchcock (19). Carefully
cleaned glassware rinsed in distilled water was used in every step of the procedure. Plastic gloves, rinsed in distilled water, were used to handle the gels and glassware. The gels were fixed overnight in 200 ml of 25 percent (vol/vol) isopropanol in 7 percent (vol/vol) acetic acid. The fixed gel was oxidized for 5 minutes in 150 ml of distilled water with 1.05 g periodic acid and 10 ml of 40 percent (vol/vol) ethanol in 5 percent (vol/vol) acetic acid (solution made up just before use). The oxidized gel was then washed eight times for 30 minutes in 200 ml of distilled water. Afterwards, the gel underwent silver staining for 10 minutes in a solution consisting of 28 ml of 0.1 N NaOH, 1 ml of concentrated (29.4%) ammonium hydroxide, 5 ml of 20 percent (wt/vol) silver nitrate, and 115 ml of distilled water. Solution should be made immediately prior to use and stirred constantly while it is being made. The stained gel was washed 4 times for 15 minutes in 200 ml of distilled water, and then developed for 10 to 20 minutes in 250 ml of developer solution (50 mg of citric acid, 0.5 ml of 37 percent formaldehyde, and a sufficient amount of distilled water to make 1 liter of solution). This solution, which should also be made immediately prior to use, should be made and used at room temperature. The developed gel was soaked for 1 hour in a stopbath (200 ml of distilled water plus 10 ml of 7 percent vol/vol acetic acid), and then finally washed with 200 ml of distilled water. The gel was then stored in plastic ziploc bags. In all steps, the gels were agitated on an orbital shaker.
$^{125}$I-surface labeling. $^{125}$I-surface labeling of CEM0 was accomplished by following the procedure described by J. Swanson (41) as modified by Hitchcock (19). Organisms, grown for 72 hours, were carefully scraped off agar plates with sterile dacron swabs and suspended in Delbecco's phosphate-buffered saline (D'PBS) (14) to a turbidity of 200 Klett units (Klett-Summerson colorimeter, blue filter, 12.5 mm path length). 1.5 ml of this suspension was placed in microfuge tubes and spun in a Microfuge B (Beckman Instruments Inc., Fullerton, CA) for 2 minutes. The supernatent was removed, and the pellet was re-suspended in 42 microliters of cold D'PBS plus 5 microliters of $1 \times 10^{-5}$ m KI. The suspension was placed in a dram vile coated with 10 micrograms of iodogen (1,3,4,6 - tetrachloro - 3, 6, phenylglycoluril) and incubated for 6 minutes at room temperature with 138.3 microcuries of $^{125}$I(carrier-free) (New England Nuclear Corp., Boston, MA). The contents of the vial were placed in a microfuge tube, the vial was washed with 600 microliters of cold D'PBS, the wash was added to the microfuge tube and spun for 2 minutes. The supernatent was removed, and the radioactive organisms were washed two additional times with 600 microliters of D'PBS. The washed pellet was resuspended in 100 microliters of 2 percent SDS lysing buffer containing 1 percent SDS (British Drug House, Poole, England), 4 percent 2-mercaptoethanol (2 ME), 10 percent glycerol, 1 m Tris(pH 6.8), and bromphenol blue. The lysate was heated at 100°C for 10 minutes with vortexing at 3 minute intervals. Ten microliters
were loaded onto 12.5 percent polyacrylamide gels (details described in SDS-PAGE section). Autoradiography of iodinated gels was done using Kodak A-R film (Kodak).

Antisera. Hyperimmune rabbit antisera raised against CEMO strain 289 was obtained as a gift from P.J. Hitchcock (Laboratory of Microbial Structure and Function, Rocky Mountain Laboratories, National Institutes of Health, Hamilton, MT). The antisera was prepared by the following procedure: (i) opaque and transparent colonies of strain 289 were suspended in sterile phosphate-buffer saline; (ii) the suspension of organisms was heated at 56°C for 1.5 hours; (iii) aliquots were frozen at -70°C; (iv) rabbits were inoculated intravenously with 0.5 ml of suspension which contained approximately $2 \times 10^8$ opaque or transparent organisms (inocula were thawed just prior to injection); (v) inoculation was repeated at 24 hour intervals; (vi) rabbits were bled days after the final injection; and, (vii) sera was stored at -70°C.

Immuno-Electroblotting. SDS-PAGE gels were subjected to electrophoretic blotting procedures using the Trans-blot apparatus (Bio-Rad Laboratories, Richmond, CA) using the Towbin buffer system as modified by Barbour. The SDS-PAGE gels were electroblotted against HAHY millipore nitrocellulose paper (NCP) for 4 hours in SDS-PAGE/NCP blot (Towbin) buffer (described below) at 18°C. After the transfer was completed, the NCP was incubated for 3 hours with 2 percent Bovine serum albumin in TSGAN (described below). The NCP was then incubated with 120 microliters
of CEMO hyperimmune rabbit antisera in 12 ml of TSGAN over-night. The NCP was washed 3 times with 50 to 100 ml of TSGAN for 20 minute intervals and then incubated with 100 microliters of $^{125}$I-Protein A in 10 ml of TSGAN for 2 hours. After incubation the NCP was rinsed once with TSEA (described below) and washed 4 times with 50 to 100 ml of TSGAN for 20 minute intervals. The NCP was rinsed many times in distilled water and allowed to dry. Autoradiography of blots was done using Kodak A-R film (Kodak).

**Blotting buffers.** The following buffers were used in all blotting procedures. SDS-PAGE/NCP Blot (Towbin) buffer (49) final concentrations: 192mM glycine; 25mM tris base; 20 percent methanol (vol/vol). TSGAN (21) final concentrations: 150mM NaCl; 5mM EDTA (formula weight 380); 50mM Tris (pH 7.4); 0.05 percent NaN$_3$ (azide); 0.25 percent gelatin and 0.05 percent Nonidet P40. TSEA (21) final concentrations: 150mM NaCl; 5mM EDTA (formula weight 380); 50mM Tris (pH 7.4); 0.05 percent NaN$_3$ (azide).

**RESULTS**

**Colony morphology.** Twenty-six phenotypes were studied in strains 48, 289, and 188. Of these 26 phenotypes, 10 phenotypes of strain 48 were designated A through J, 6 phenotypes of strain 289 were designated K through O, 10 phenotypes of strain 188 were designated P through V dark. All phenotypic differences were observed by stereomicroscopic examination. To be consistent, all observations were made on 96 hour cultures. All findings are summarized in Tables 1, 2, and 3.
In strain 289, four very translucent colonial variants were isolated. These variants are phenotypes L, M', N, and O (Table 2). When examined in illuminated light, these colonies are metallic gold (fig. 1a). In reflected light, all colonies are gold (fig. 1b). The consistency of the 4 phenotypes varied from friable to butyrous.

In all three strains, colonial variants were identified that are of intermediate opacity (translucent) (Tables 1-3). These colonies range from frosted white to lightly frosted gold in color when observed in transmitted light (fig. 1c). When examined in reflected light, these colonies range from gold to dark gold (fig. 1d).

Eight colonial variants were identified as having opaque colonies. These opaque colonial variants are: phenotypes A and H of strain 48; phenotype K of strain 289; and phenotypes T', Q, R, S, and T of strain 188 (Tables 1-3). These colonies are frosted gold in color when observed in transmitted light (fig. 1e), and dark gold in color under reflected light (fig. 1f).

The size of the colonies at 96 hours range from those colonies which are approximately 5 mm in diameter to colonies which are 10 mm in diameter. Although detailed measurements of the colony diameter at 96 hours of each phenotype has not been done at this time, there are a few phenotypes whose colony morphology is identical except for the diameter of their colonies. Phenotype L (translucent) colonies are one-half the size of
colonies from phenotype N (translucent). Colonies from phenotypes E and V'(intermediate) are one-half the size of colonies from D and V'dark, respectively. Also, phenotype T (opaque) colonies are twice the diameter of phenotype T' (opaque) colonies.

The three opaque phenotypes A(48), K(289), and Q(188) exhibit an interesting phenomenon. The majority of the colonies in each culture have entire borders with one or more processes (fimbria) projecting from their edges (fig. 2). The number of colonies that are fimbriate in a culture varies greatly from generation to generation. Attempts to passage these colonies as an entire culture have been unsuccessful.

The variants of all 26 phenotypes are very stable. Phenotypic differences in colony morphology are detectable at 24 hours of growth and do not change as the colonies age. Also, no changes in streptomycin sensitivity were observed (data not shown).

**SDS-PAGE.** Whole Cell (WC) lysates and Proteinase K (PK) digests of all 26 phenotypes were electrophoresed in 12.5% polyacrylamide gels. The fixed gels were stained with CBB and a silver stain which stains lipopolysaccharide (LPS) and protein. Fig. 3 is a photograph of CBB gels containing WC lysates. The major stained band (M) is approximately 41 K daltons apparent molecular weight (Mr). In the CBB gels this band is a heavy dark blue band; in the silver stained gels (fig. 4) this band is a yellow and brown doublet. The major band (M) is irregular in all phenotypes except phenotypes E, F, and I of strain 48.
In the CBB stained gels, a predominantly stained band (+) of approximately 100 K daltons Mr is seen in strains 289 and 188, but not strain 48. On the silver stained gels (fig. 4), a tan colored band (\(\Theta\)) of approximately 20 K daltons Mr stains clearly in strains 48 and 289, but not in strain 188. A dark brown band (\(\Phi\)) of approximately 16 K daltons apparent Mr is found in all phenotypes of 188 except phenotype 0. This band is also detected in phenotype C of strain 48. The low molecular weight (MW) brown bands (\(\text{[]}\)) of phenotypes E, F, I, and Q are different from the low MW brown bands of the phenotypes in their respective strains. No major differences are seen in these low MW bands in the phenotypes of strain 289.

In the silver stain gels loaded with PK digest, a broad diffuse yellow band is seen in all phenotypes except phenotypes E, F, and I of strain 48 (data not shown). This broad diffuse band is not observed in PK digest gels that were stained with CBB. Also, this band correlates with the broad band of antigenic material in the PK digest loaded lanes of the Immuno-electroblots (fig. 6). Also, in both CBB and silver stained gels loaded with PK digest, there are bands which partially stained to varying degrees.

Preparations of WC lysates of these phenotypes made 6 months apart were compared in polyacrylamide gels. No differences were seen in the gel profiles. This evidence is consistent with observations on colonies - the phenotypes of this organism seem to be stable.
I₂⁻Surface labeled proteins. The surface proteins of 9 phenotypes were labeled with I₂⁻ using the procedure in Material and Methods. The phenotypes labeled were: A, B, E, F, and I from strain 48; K, and M from strain 289; and, Q and V dark from strain 188. In fig. 5 a predominantly labeled band (+') that has an apparent MW of approximately less than 100 K daltons is seen in strains 289 and 188, but not in strain 48. This band has the same Mr as the 100 K dalton band (+) seen in CBB gels. The major protein band (M) of CEMO appears on the autoradiogram, but it is not labeled as well as the less than 100 K dalton band (+). A band of approximately 19 K daltons Mr (●) is clearly seen in phenotypes A, B, K, and M. It is faintly seen in phenotypes Q and V dark, and not seen at all in phenotypes E, F, and I. Also, phenotypes E, F, and I are not labeled as well as the other phenotypes, even though the CBB gel demonstrates that all WC lysates are present in similar amounts (data not shown).

Immuno-Electroblotting. WC lysates and PK digests of all 26 phenotypes were immunoelectroblotted as described in Method and Materials. The WC lysates and PK digests of each phenotype were loaded into adjacent lanes in the same gel (fig. 6). In the lanes loaded with WC lysate there is a broad diffuse band (●) that is seen in strains 48, and 188. In strain 289, this band does not appear, but a broad diffuse band (●') that closely resembles it is seen with a slightly higher MW.
A very large diffuse band of antigenic material (▼) is seen in all lanes loaded with PK digest except those lanes loaded with PK digest of phenotypes E, F, and I. The major staining band of CEMO (M) is not recognized by the antibody-protein A complex.
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<th>Reflected Color</th>
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</table>

(a) s = sensitive  
(b) O = opaque  
(c) Op/Tr = intermediate  
(d) V = very translucent  
(e) F = colonies are fragile but become butyrous with age  
(f) O/F = intermediate fragile
TABLE 2 - STRAIN 289 COLONY MORPHOLOGY

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(d) Intermediates are very translucent but become butyrous with age
(e) Intermediates are intermediate but become butyrous with age
(f) Opacity - Opaque: Opaque; TR/OP: Intermediates; TR/OP = Very translucent
(g) S = Sensitive
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<td>☑️</td>
<td>☑️</td>
<td>Gold</td>
</tr>
</tbody>
</table>

TABLE 3 - STRAIN 188 COLONY MORPHOLOGY

(d) Intermediates - colonies are visible but become buttonous with age
(e) Intermediates - colonies are intrusive but become buttonous with age
(f) Opacity - O = opaque; Op/Tr = Intermediate; Tr/Op = Very transparent
(g) R = Resistant

---

- Gold: gold hue, Protrate, white with gold, gold.
- Intermediate: intermediate, Lightly frosted gold.
- Occasional: occasional, Lightly frosted gold.
- Consistency: consistency, Transparent, Sheen, Gold.

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- Consistency: Intermediate, Occasional
- Borders: yes, yes
- Opacity: yes, yes
- Reflectivity: yes, yes
- Transmittance: yes, yes
- Color: Gold

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- Strain 188 Colony Morphology Table

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- TABLE 3 - Strain 188 Colony Morphology

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- Consistency: Intermediate, Occasional
- Borders: yes, yes
- Opacity: yes, yes
- Reflectivity: yes, yes
- Transmittance: yes, yes
- Color: Gold
Fig. 1a - Strain 48, Opaque colonies under transmitted light
Fig. 1b - Strain 48, Opaque colonies under reflected light
Fig. 1c - Strain 48, Intermediate colonies under transmitted light
Fig. 1d - Strain 48, Intermediate colonies under reflected light
Fig. 1e - Strain 289, Translucent colonies under transmitted light
Fig. 1f - Strain 289, Translucent colonies under reflected light

Fig. 2 - Strain 48, Phenotype A colonies that exhibit the occasional fimbriate processes projecting from edge of the colonies
Fig. 3 - CBB stained gel of CEMO WC lysates
Fig. 4 - Silver stained gel of WC lysates
Fig. 5 - $^{125}$I-surface labeled proteins of CEMO
Fig. 6 - Immuno-electroblot autoradiograms of CEMO MC lysates and PK digests.

$+$ = WC

$X = PK$
DISCUSSION

The data presented in these studies shows that there are molecular differences among these three CEMO strains. The protein band of less than 100 K daltons Mr that is found in CBB gels and $^{125}$I-surface labeled protein and autoradiograms. This band is present in strains 289, and 188, but not seen in strain 48. The presence of this band correlates with the origin of each strain. Strains 289, and 188 were isolated in Kentucky and strain 48 was isolated in Germany. Another example is the tan band of 20 K daltons Mr in the silver stained gels. This band is present in strains 48 and 289, but not in strain 188. This band is very interesting because its presence correlates with the streptomycin sensitivity while 188 is streptomycin resistant. The dark diffuse band (©) that is seen in the immuno-electroblots of strains 48 and 188 and the dark diffuse band (©') in strain 289 that resembles the band in 48 and 188 represent another major variation between the strains.

The molecular differences among these three strains are important in their study. Microorganisms vary in all characteristics that are used in their identification and differentiation. This variation depends on the genetic control mechanisms and/or their functions and the environment the organism grows in. These two influences cause organisms to produce certain types of intracellular structures and enzyme systems that make them unique from different organisms. The differences in the molecular composition between two microorganisms are frequently
associated with their differences in physiological characteristics of different organisms and could correlate certain characteristics to certain stained bands. Ultimately one can discover which genes control certain characteristics.

The major stained band (M) is seen in all 26 phenotypes of CEMO investigated. This band is protein because it stains prominently in CBB gels. However, this band does not label very well by the iodogen method or by immunoelectroblotting. 125I will label surface proteins if they have histidine-tryptophan sequences in their primary sequence. Thus, the major stained band (M) appears not to consist of a large percentage of histidine-tryptophan sequences. The lack of prominent labeling by the major stained band (M) may be due to the poor transfer of the protein from the gel to the nitrocellulose paper. Further investigations into these situations are needed.

The low molecular weight PK resistant brown bands seen in both the whole cell lysate and PK digest silver stains resemble the LPS profiles of Salmonella minnesota rough mutant R 345. These bands are similar to the silver stained profile of phenol-water (putative LPS) of CEMO (Hitchcock, unpublished data). Also, Hitchcock and Brown have shown that silver stained profiles of PK digested WC lysate are similar to homologous purified LPS (19). Phenotypes E, F, I, and Q have presumptive LPS profiles with major differences in them when compared to the other phenotypes. These differences could indicate differences in the way these phenotypes interact with their equine hosts.
Not only do E, F, and I differ from the other phenotypes in their presumptive LPS profile, they differ in other ways. In the CBB and silver gels, the major stained band \((M)\) is distorted except in phenotypes E, F, and I. A band of approximately 19 K daltons MW \((\bullet)\) is seen in the \(^{125}\text{I}\)-surface labeled autoradiograms in all phenotypes investigated except E, F, and I. Silver stained gels and immunoelectroblot autoradiograms of PK digests show a very broad diffuse band in all phenotypes except E, F, and I. This band is probably not protein because it does not appear in PK digest gels stained with CBB. It may be that the broad diffuse band causes the irregularities in the major stained band \((M)\). Further studies are required to analyze this interaction and to determine what this band is.

Colonial morphology is in part a function of the morphology of the individual cells making up that colony. Also, the colonial differences have been associated with other significant characteristics of a bacterium such as virulence, antigenicity, and immunogenicity. Pathogenic types of Neisseria gonorrhoeae are thought to differ from non-pathogenic types in their piliation. Virulence of anthrax bacillus can be determined by determining the colonies smooth or rough appearance. Pathogenic forms of Salmonella sp. with smooth LPS can be distinguished by their colony morphology also.

The morphology of CEM bacterial colonies were previously observed by Sahu et al. (37). He reported the observation of
4 morphological types (smooth, sandy, colony with rings, colony with blebs) grown on Eugon agar, and 1 morphological type (large smooth colonies) grown on Tryptose blood agar. Sahu described these 5 morphological types by their size, shape, and general appearance. Observations on color, opacity, and consistency were not recorded. Since ECA and TrCA medium is not clear and accurate observations on color and opacity are not possible.

In this investigation CEMO was grown and observed on clear media. All colonies were smooth and entire; no rough forms were isolated. Sandy colonies, colonies with rings or blebs were not observed in 96 hour cultures. With the additional observations on color under reflected and transmitted light, opacity, and consistency, 26 morphological phenotypes were recorded. These 26 phenotypes could be distinguished into 3 major colonial variants based on opacity. Among these variants, few differences in gel patterns could be detected. Phenotypes E, F, and I were all of intermediate opacity, but not all intermediate opacity phenotypes had gel profiles similar to E, F, and I.

An interesting characteristic is the fimbriate colonies on phenotypes A, K, and Q. This characteristic is not always present on the colonies in the phenotypes that exhibit this. There may be some condition that is unknown that triggers the appearance of this characteristic. This occasional fimbriation could be caused by motile cells streaming away from the colony or
some disruption of the adherence of the bacteria at the edges of the colony.


