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A Sero-epidemiological Study of
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A SERO-EPIDEMIOLOGICAL STUDY OF THE CAUSATIVE AGENT OF CONTAGIOUS EQUINE METRITIS

By

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ABSTRACT

A SERO-EPIDEMIOLOGICAL STUDY OF THE
CAUSATIVE AGENT OF
CONTAGIOUS EQUINE METRITIS

By

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This study examined several species, horses, cattle, sheep, pigs, dogs and humans, for serological evidence of exposure to the causative agent of contagious equine metritis. Serum samples are tested by serological test methods developed for detection of the contagious equine metritis organism (CEMO) in horses. The methods used are a plate agglutination test, a complement fixation test and a passive hemagglutination test.

The serological methods are compared for their correlation of test results and technical merit as an epidemiological screen.

The morphology of the streptomycin sensitive strain of the CEMO is studied by electron microscopy.

Serological test results indicate that sheep, pigs and cattle may have exposure to CEMO or related organisms. There is a statistically significant increase in the incidence of antibodies to CEMO in human patients being tested or treated for venereal disease.

Of the serological tests evaluated, the passive hemagglutination method is the best suited for epidemiological studies.

TO JOHN

Your love, understanding and encouragement
has made this possible.

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INTRODUCTION

Contagious equine metritis (CEM) is a recently discovered venereal disease of horses. Although CEM is a highly contagious disease, it is not a serious infection for the horse itself. The primary impact of this disease is due to the serious economic effect that delays in successful breeding have on the Thoroughbred industry.

Contagious equine metritis is caused by a previously unknown, fastidious, Gram-negative coccobacillus which has not yet been placed into a bacterial taxonomic classification. A foremost question when any new bacterium is isolated from one host, especially if pathogenic, is whether it is pathogenic for other animal species, especially man. The primary impetus for this research project is the identification of other animal species which might have had exposure to or have been a reservoir of the CEM organism.

Serological test results indicate that sheep, pigs and cattle may have exposure to CEMO or related organisms. There is a statistically significant increase in the incidence of antibodies to CEMO in human patients being tested or treated for venereal disease.

CHAPTER I

LITERATURE REVIEW

1.1. HISTORICAL REVIEW

Contagious equine metritis (CEM) was first reported in 1977 by Crowhurst (1977). That outbreak involved Thoroughbreds at the National Stud in Newmarket, England. The highly contagious nature of this disease was soon evident and breeding was stopped at the National Stud in an effort to control the spread of infection. However, reports of other outbreaks in the Newmarket area soon appeared (Platt et al. 1977, Ricketts et al. 1977). Investigation revealed a clinically similar metritis had been present in Ireland in 1976 (O'Driscoll 1977). By 1980, CEM was confirmed in eight countries. The disease was spread mainly through the importation of clinically healthy Thoroughbred breeding stock.

The clinical picture in CEM is an endometritis with an associated inflammation of the cervix and vagina. Most infected mares fail to conceive, although there is no permanent infertility. Mares have no signs of systemic involvement. Stallions show no signs of genital infections and are passive carriers (Platt et al. 1977, Ricketts et al. 1977). Transmission is primarily venereal; however CEM is highly contagious and has been transmitted during examination and handling by breeding shed personnel (David et al. 1977).

Many difficulties were encountered in isolating the causative agent of CEM. Platt et al. (1977) succeeded in culturing a Gram-negative coccobacillus from cervical swabs taken from mares with acute infections. This organism was fastidious, requiring enriched media and increased CO_2 for initial isolation, and was morphologically similar to organisms observed in the smears of uterine exudate from infected mares. Transmission studies demonstrated that this newly isolated bacterium caused CEM (Platt et al. 1977, Ricketts et al. 1977). The contagious equine metritis organism (CEMO) has not yet been placed into any bacterial taxonomic classification, although Taylor et al. (1978) have proposed the name of Hemophilus equigenitalis.

In September of 1977, a code of practice was published in England which listed recommendations for the control of CEM (David et al. 1977). This included the establishment of a bacteriological screening program for mares and stallions and specific improvements in hygiene standards on stud farms. It also contained recommendations for the treatment of infected horses. Other involved countries developed similar standards.

The effectiveness of the code in England was substantiated by the reduction of cases in 1978. By the end of the 1977 breeding season, approximately 200 mares were infected at 29 breeding farms, involving 23 stallions (Powell 1978). In 1978 there were 2 outbreaks of CEM involving 4 mares and 2 stallions (Powell and Whitwell, 1979). The bacteriological screen detected 48 infected mares prior to breeding.

1.2. BACTERIOLOGICAL CHARACTERISTICS OF CEMO

1.2.A. Morphology

The CEMO is usually described as a Gram-negative coccobacillus, but they may appear as short rods with bipolar staining resembling members of the Brucella and Pasteurella genera (Eaglesome and Garcia 1979). Pleomorphic forms may appear after prolonged incubation. The organism is non-motile and no flagella were observed by electron microscopic studies. On 10% chocolate agar, the organism produced tiny, round and raised, greyish-white colonies approximately 0.5 mm in diameter after 48 hours incubation at 37°C (Eaglesome and Garcia 1979, Sahu and Dardiri 1980).

1.2.B Cultural and Biochemical Characteristics

The CEMO is a fastidious organism requiring enriched media and a 5-10% CO_2 atmosphere. Initial reports claimed a microaerophilic atmosphere of 5-10% CO_2 in hydrogen was required (Platt et al. 1977, Ricketts et al. 1977) but others obtained growth in 5-10% CO_2 in air (Taylor et al. 1978, 1979). Growth occurred over a temperature range of 30-41°C with optimum growth at 37°C. Best growth of the CEMO was obtained on chocolate agar made with 10% horse blood and Eugon agar, incubated under 5-10% CO_2 at 37°C (Taylor et al. 1978).

The bacterium is catalase, cytochrome oxidase and phosphatase positive but unreactive in other conventional tests for biochemical characterization. The CEMO does not ferment carbohydrates nor requires X (hemin) or V (NAD) factors. The lack of dependence on X factor was confirmed by a positive result with the D-amino-levulinic acid test of

Kiliam (Taylor et al. 1978). However, CEMO growth is stimulated by X factor.

Taylor et al. (1978) have determined the DNA base composition, as estimated by melting point temperature, to be 36.1 mole per cent guanine plus cytosine (G+C).

The CEMO appears to be sensitive to many antibiotics including benzyl-penicillin, ampicillin, tetracyclines, trimethophrim, sulfamethoxazole, clindamycin, polymyxin B, furazolidone, and trivetrin (Taylor et al. 1978, Eaglesome and Garcia 1979). Strains of CEMO isolated in England and Ireland were resistant to streptomycin but a strain of CEMO isolated in Kentucky was found to be sensitive to streptomycin (Swerczek 1978a).

1.2.C. Serological Testing

Cross-agglutination studies were performed to identify antigenically similar and possibly related bacteria. Serological tests were performed by several investigators, using antisera to other known Gram-negative bacteria and purified anti-CEMO antibodies induced in rabbits (Taylor et al. 1978, Smith 1978). The CEMO did not react with various Brucella antisera in slide agglutination tests. A variety of known Gram-negative bacteria were tested against the anti-CEMO antisera. Smith (1978) reported consistent cross-reactivity with Moraxella and Mima species. Taylor et al. (1978) reported cross-reactivity with Hemophilus, Moraxella, Neisseria, and Pasteurella species.

1.2.D. Taxonomic Classification

Establishment of taxonomic groups in bacteria is difficult. Bacteria multiply asexually and thereby lack the means for genetic exchange which leads to the evolution of distinct species. Instead, mutations accumulate which produce gradients of related types. Bacterial groups of related ecologic types are easily recognized. However, division of these groups into species becomes a decision as to what extent 2 types must differ before they are classified as distinct species. Molecular composition of DNA has now been added to the taxonomic criteria as an objective measure of the relationship between bacteria. Closely related bacteria must have similar G+C mole % ratios, although such similarities are not proof of a close relationship.

Characteristics of CEMO and serologically similar bacteria are listed in Figure 1. Data for the CEMO is from Taylor et al. (1978) while characteristics for other bacterial genera are compiled from Bergey's Manual of Determinative Bacteriology (Buchanan and Gibbons 1974). Taylor et al. (1978) proposed the name of Hemophilus equigenitalis for CEMO. However, CEMO lacks dependency on either X or V factors, characteristics many consider primary requirements for inclusion into the Hemophilus group. The CEMO does not appear to be closely related to Pasteurella, which is much more biochemically active than CEMO, producing acids from sugars, H_2S and reducing nitrates. The remaining bacterial groups, Neisseria, Branhamella, Moraxella and Acinetobacter all belong to the Neisseriaceae family, which is presently undergoing taxonomic revision (Baumann et al. 1968a, 1968b; Henriksen and Bøvre 1968). The CEMO has many characteristics in common with the Neisseriaceae family, and

CHARACTERISTIC	CEMO	HEMOPHILUS	PASTEURELLA	NEISSERIA	BRANHAMELLA	MORAXELLA	ACINETOBACTER
SHAPE	CB	CB	CB	C	C	CB	CB
PRODUCES ACIDS FROM SUGARS	–	V	+	D	–	–	D
CYTOCHROME OXIDASE	+	NA	+	+	+	+	–
REDUCES NITRATES	–	+	+	D	+	D	–
G+C MOLE PER CENT	36[a]	38 - 42	36 - 43	47 - 52	40 - 44	40 - 46	39 - 47
FACULTATIVE ANAEROBE	+	+	+	+	+	–	–
PENICILLIN SENSITIVE	+	+	+	+	+	+	–
PARASITE OF MAMMALIAN MUCOUS MEMBRANES	+	+	+	+	+	+	–

DATA FOR CEMO FROM TAYLOR ET AL. (1978)

DATA FOR OTHER BACTERIAL GENERA COMPILED FROM BERGEY'S MANUAL OF DETERMINATIVE BACTERIOLOGY, 8TH ED., BUCHANAN AND GIBBONS (1974)

LEGEND

NA	INFORMATION NOT AVAILABLE
+	MOST (GREATER OR EQUAL TO 90%) POSITIVE
–	MOST (GREATER OR EQUAL TO 90%) NEGATIVE
V	STRAIN INSTABILITY, NOT DIFFERENCES BETWEEN STRAINS
D	SOME STRAINS POSITIVE, SOME STRAINS NEGATIVE
C	COCCOID SHAPE
CB	COCCO-BACILLARY SHAPE
a	G+C MOLE PER CENT DETERMINED BY THE TEMPERATURE MELTING POINT, ALL OTHERS DETERMINED BY THE BUOYANT DENSITY METHOD

FIGURE 1. CHARACTERISTICS OF CEMO AND SEROLOGICALLY SIMILAR BACTERIAL GENERA

I believe that it belongs in that group.

The recognized species of Acinetobacter are separated from Moraxella based on their lack of cytochrome oxidase. Acinetobacter species are usually saprophytic and resistant to penicillin. It is doubtful that the CEMO belongs in this genus.

Neisseria, Branhamella and Moraxella are known parasites of mammalian mucous membranes. They are biochemically inert and sensitive to penicillin. Both Neisseria and Moraxella are known agents of genital infections in man. The G+C ratio for Neisseria is 47-52%, which is significantly higher than that determined for the CEMO. However, the G+C ratio for Branhamella and Moraxella, 40-46% by the buoyant density method, is close to the CEMO ratio, 36% by the melting point method. This small difference in G+C ratios could arise from differences between methods used in determining the G+C mole per cent.

Moraxella and Branhamella are phenotypically and genetically very similar and it has been proposed to fuse the two into one genus (Henriksen and Bøvre 1968). This proposal has not been universally accepted since the genera differ in their gross morphology. Although the significance of shape is unknown, it has a long-standing historical value. However, in some bacteria, morphology is dependent on the phase of the growth cycle. For example, Acinetobacter are definitely bacillary during their exponential growth phase, becoming shorter and more coccoid in appearance when viewed in the other growth phases. The CEMO also has this phase-dependent morphology, as demonstrated by finding predominantly bacillar forms on media which support good growth of the CEMO, and predominantly coccoid forms on less supportive media (Swaney and Breese,

1980). Taxonomic division based on morphology is not suitable for such types of bacteria. Thus the division between Moraxella and Branhamella on the basis of morphology may be artificial.

The known characteristics of CEMO do not allow placement into any established taxonomic classification. Many characteristics suggest a close relationship with Branhamella and Moraxella, genera which are presently undergoing taxonomic revision. Thus final placement of the CEMO must be postponed.

1.3. DIAGNOSTIC TESTS FOR CEM

1.3.A. Bacteriological Screen

The bacteriological screen, established by David et al. (1977) includes three sets of swabs taken from several sites at weekly intervals. At least one set must be taken from mares during estrus. Stallions and teasers have swabs taken from the urethra, urethral fossa and prepuce. Samples of pre-ejaculatory fluid are also cultured. Mares have swabs taken from the endometrium, cervix and clitoral fossa including the clitoral sinuses. All three sets of cultures must be negative before the horses can be bred. Careful screening has determined that CEMO can persist in the clitoral sinuses after treatment and eradication of the organism from the rest of the genital tract (Simpson and Eaton-Evans 1978).

1.3.B. Serological Screens

Serological tests which have been developed for CEM detection in horses include:

1. A serum agglutination test with antiglobulin phase (SAT), developed by Benson et al. (1978).
2. Two complement fixation methods (C-FIX), one developed by Croxton-Smith et al. (1978) and the other by Bryans et al. (1979).
3. A passive hemagglutination test (PHA), developed by Fernie et al. (1979).
4. A plate agglutination test (PAT), developed by Swerczek (1979, 1980).
5. An enzyme-linked immunosorbent assay (ELISA), developed by Sahu et al. (1979).

All test methods found relatively high serum antibody titers in mares with active CEM infections and diagnostic titers have been established for each test method. Specific information on these tests will be provided later.

1.3.C. Problems in CEM Diagnosis

The primary problem in the control of CEM is the detection of clinically healthy carrier mares. Bacteriological methods are not reliable for several reasons. The first problem is the low number of organisms present in the carrier state. More importantly, CEMO is a slow growing, fastidious bacterium, often requiring several days to a week for primary isolation on enriched media, allowing more rapidly growing normal genital flora to obscure other colonies on the culture plate. The original strains of CEMO isolated in England and Ireland were streptomycin resistant, and subsequently, streptomycin was used in the primary isolation media to inhibit the growth of most normal flora. However, in 1978,

a streptomycin sensitive strain of CEMO was found in Kentucky (Swerczek 1978a). It was also determined that certain organisms among the normal genital flora produce a metabolite which inhibits the growth of CEMO (Swerczek 1978b, Altherton 1978). These combined factors make the identification of a streptomycin sensitive CEMO carrier mare by bacteriological methods quite difficult.

Serological tests demonstrated relatively high serum antibody titers in mares with active CEM infections. However, antibody levels in carrier mares were often below diagnostic levels. Stallions do not have demonstrable CEM titers because they do not become infected with CEMO but are simply passive carriers of the organism. Thus the present serological tests for CEM are not useful for detection of infected stallions or carrier mares (Benson et al. 1978, Croxton-Smith et al. 1978, Fernie et al. 1979, Swerczek 1978a, 1979, 1980).

1.3.D. Comparison of Screening Methods

There are many advantages in using serological over bacteriological screening tests. Serological samples are easier to obtain and are more stable. The samples can be obtained at any time, independent of the mare's reproductive cycle. Serological tests are less labor intensive than culture techniques and test results can be obtained much faster. Most serological tests can be completed within 2 days while culture results can be called negative only after 6 days incubation (Anon., Vet. Rec., 1980).

The major disadvantage of serological tests is that they give only presumptive results. True confirmation of bacterial infection can only be

achieved by actual recovery of organisms from the infected site. The immune response of antibody production starts after antigen exposure and usually takes several days to occur. Thus a serum sample taken very early in an infection would yield negative results when culture techniques may have been able to detect the organism. Also serological tests may remain positive after resolution of the infection.

1.4. SEROLOGICAL TECHNIQUES FOR CEM DETECTION

1.4.A. General Review

Serology is, by definition, the study of in vitro antibody-antigen reactions. These reactions may be demonstrated by a variety of techniques, depending mainly on the nature and structure of the antigen. The antibody-antigen reaction itself is affected by many factors, including time, temperature, electrolyte concentration, pH and ratio of antigen to antibody.

Many techniques are available to measure antibody levels, with varying degrees of specificity and sensitivity. Antibody-antigen reactions are by their very nature specific. Serological test specificity refers to the degree of accuracy with which one can detect the desired agent, be that antigen or antibody. Sensitivity refers to the level of detectable antibody-antigen complexes.

Most antibody-antigen reactions occur in two distinct stages. The first is the specific combination of antibody and its corresponding antigen or hapten. The second stage is the formation of an observable reaction such as precipitation, agglutination or complement fixation. These two stages often overlap, each stage requiring a different time

span, temperature, electrolyte concentration or other specific condition. Antibody-antigen reactions may also occur without the development of an observable reaction.

1.4.B. Diagnostic Test Considerations

i. Antibody Detection. Serological tests used to measure the humoral immune response (antibody production) fall into three categories. The most sensitive are the primary binding tests that directly measure the amount of immune complex formed (Tizard 1977). Competitive radioimmunoassays are in this category and can detect antibody concentrations as low as 5×10^{-5} µg protein/ml. Secondary binding tests measure the consequences of immune complex formation in vitro. These tests are less sensitive than primary binding tests, but are considerably simpler to perform. The types of measurable antibody-antigen interaction include precipitation of soluble antigens, agglutination of particulate antigens, and activation of complement. The sensitivity of secondary binding tests varies with the procedure, requiring from as little as 5×10^{-5} µg protein/ml to as much as 3×10^{1} µg protein/ml for an observable reaction. Tertiary tests measure the consequences of the immune response in vivo. An example is passive cutaneous anaphlyaxis, with a sensitivity of 2×10^{-2} µg protein/ml (Tizard 1977).

ii. Antibody Quantification. Most serological tests are semi-quantitative. While a simple positive or negative result is sufficient for some tests, it is usually necessary to estimate the amount of antibody present for diagnostic purposes. The serum titer, a common unit of comparison in serological tests, is defined as the reciprocal of the

highest dilution of serum yielding a positive reaction. The serum dilution itself is also used as a comparison unit. A common problem in interpretation of both systems is whether the unit refers to the dilution of serum used in the test procedure or the actual final dilution of serum in the test reaction. The SAT method of Benson et al. (1978) refers to the actual final dilution of serum in the test reaction while the PHA method of Fernie et al. (1979), the ELISA method of Sahu et al. (1979), and the two C-FIX methods of Byrans et al. (1979) and Croxton-Smith et al. (1978) refer to the dilution of serum used in the test proper.

While these methods of estimating antibody levels are adequate for diagnostic purposes, they are not a true measure of antibody concentration. The demonstrable reactivity of a serum sample is dependent on the immunoglobulin class of antibody present as well as the concentration. The immunoglobulin class responsible for the reaction is especially critical in secondary binding tests since different classes have varying ability to form the secondary reactions of precipitation, agglutination or complement fixation. Thus, secondary binding methods can over- or underestimate antibody levels (Tizard 1977).

iii. Immunoglobulins of Test Species. Five classes of immunoglobulins are known, designated as IgG, IgM, IgA, IgD, and IgE. All of the domestic animals possess IgG, IgM, and IgA. It is probable that most possess IgE, although it has not been isolated in all species. IgD has been found only in man, and its function is not well understood. The basic characteristics of these immunoglobulin classes do not differ between species. However the number and types of subclasses do vary between species (Tizard 1977).

Immunoglobulin G. IgG is the immunoglobulin class found in the highest concentration in the serum (Tizard 1977). It plays a major role in antibody-mediated defense mechanisms. Its small size allows passage through blood vessels to tissue spaces and body surfaces. IgG can opsonize, agglutinate and precipitate antigen. It can activate complement only if sufficient numbers have accumulated in a specific configuration on the antigen (Delaat 1974).

Immunoglobulin M. IgM is found in the second highest concentration in the serum of most animals (Tizard 1977). It is the major immunoglobulin produced in a primary immune response. IgM is also produced in a secondary response, but is often masked by the massive IgG production. On a molar basis, IgM is considerably more efficient than IgG at complement fixation, opsonization, agglutination, and viral neutralization (Delaat 1974). Due to its large size, IgM is confined to the blood vascular system.

Immunoglobulin A. IgA is found in the second highest concentration in man, though it is a minor component in animal serum (Tizard 1977). It is the major immunoglobulin found in external secretions. As such, its primary function is to protect the exposed environs of the gastrointestinal, respiratory and genital tracts, the udder and eyes. IgA cannot activate complement or act as an opsonin. It can agglutinate particulate antigens and neutralize viruses.

Immunoglobulin E. IgE is in extremely low concentrations in the serum and has not been found in all species (Tizard 1977). IgE mediates Type I hypersensitivity reactions seen in allergies and anaphylaxis. It plays no known role in serological testing.

1.4.C. Serological Tests for CEM Diagnosis

i. Serum Agglutination Test (SAT). The SAT for CEM developed by Benson et al. (1978), relies on the visible agglutination of the CEMO antigen. The SAT antigen is a boiled saline suspension of CEM bacteria. Antigen and diluted serum are incubated together at 37°C and the degree of bacterial agglutination determined. Agglutination of more than 25% of the bacterial antigen is a positive test reaction. Species specific antiglobulin sera is added to the washed test mixture to allow detection of non-agglutinating antibodies bound to the CEM antigen. The use of antiglobulin sera increases test sensitivity and clarifies most inconclusive SAT test results.

Serum samples from unexposed horses had SAT titers up to 20. Titers greater than or equal to 80 were considered positive. Samples with titers of 40 were considered inconclusive with SAT and further tested with equine antiglobulin sera. If the titer with antiglobulin sera remained the same as the SAT result, the sample was considered negative. If the titer increased, the sample was considered positive.

ii. Plate Agglutination Test (PAT). Plate agglutination tests rely on the rapid formation of visible agglutination patterns when the antigen preparation is mixed with serum containing the corresponding antibody. Swerczek (1978a, 1979, 1980) developed a PAT for CEM detection in horses. The antigen preparation is a stable suspension of intact CEM bacteria. Two test procedures are available, depending on the breed of horse being tested. The Thoroughbred procedure requires a stronger agglutination pattern for a significant positive test result and uses less serum than the non-Thoroughbred procedure. Test results of complete

agglutination (4+) with sera diluted 1:2 were considered positive for CEM. Lesser reactions were recorded but the significance of these reactions are unknown (Swerczek 1979).

iii. Complement Fixation (C-FIX). Complement fixation tests are based on the ability of certain antibodies to bind and activate (fix) complement. Antibodies can fix complement only after binding to an antigen. Thus, the amount of activated complement is proportional to the amount of antigen bound antibody. Sheep red blood cells are readily lysed by activated complement and are used as an indicator of complement activity.

Complement fixation tests consist of two distinct stages. In the first (test) stage, serum and the specific antigen are mixed together in the presence of complement. Interaction of serum antibody and specific antigen may fix complement. In the second (indicator) stage, antibody coated sheep erythrocytes (RBCs) are added to the test mixture. The antibody coating the sheep RBCs will activate any remaining complement and cause red cell lysis. Thus, the amount of red cell lysis is proportional to the excess of complement over the amount of specific antibody present in the test stage. Antibody coated sheep RBCs and complement activity must be carefully balanced so that complement fixation in the test stage will prevent lysis of the coated RBCs in the indicator stage. All reagents and serum samples are also tested for their ability to non-specifically absorb complement in the absence of antigen.

The specific C-FIX methods developed by Bryans et al. (1979) and Croxton-Smith (1978) are similar in that they both use a saline

suspension of intact CEM bacteria as the antigen. Bryans et al. (1979) showed that the reactivity of the CEMO antigen preparation is related to the proportion of organisms with intact bacterial envelopes. Both methods have established the test reaction of 4+ (no lysis) in the 1:4 dilution of serum as the minimal positive test result in horses. Croxton-Smith et al. (1978) had a high percentage of samples with anti-complementary activity, 26% AC, although Bryans et al: (1979) did not report such findings. Anticomplementary activity invalidates C-FIX test results and decreases the usefulness of the method for diagnostic purposes.

iv. Passive Hemagglutination (PHA). Erythrocytes that are treated with dilute tannic acid will adsorb proteins onto their surfaces (Boyden 1951, Stavitsky 1954). Using RBCs as inert carriers of various antigens aids in the visualization of antibody-antigen reactions in test systems. By this method, many precipitation reactions can be converted into more sensitive agglutination test systems (Boyden 1951, Stavitsky 1954, Delaat 1976). Serum samples used in PHA tests must have antibodies to the carrier system removed prior to testing for the added specific antigens. This is accomplished by incubating serum samples with tanned but uncoated carrier cells. This adsorbed serum is used in subsequent PHA tests.

Fernie et al. (1979) developed a passive hemagglutination test for CEM. This test method differs significantly from the previously described tests in that it uses soluble CEMO antigens rather than intact bacteria. A saline suspension of CEMO is disrupted by ultrasonic vibrations and the resulting cellular debris is removed by centrifugation.

Formolized (Sequeira and Eldridge, 1973) and tanned (Boyden 1951) turkey erythrocytes are used as carriers for the antigen preparation. Diagnostic PHA serum titers for CEM in horses have been established. Titers up to and including 32 were considered negative for CEM exposure. Titers of 256 or higher were considered positive. Results between 32 and 256 were suspicious for CEM exposure but inconclusive (Fernie et al. 1979).

v. Enzyme-linked Immunosorbent Assay (ELISA). Enzyme immunoassay is a recently developed quantitative serological technique which is analogous to radioimmunoassay and quantitative immunofluorescence. Advantages of ELISA include sensitivity (ng/ml range), simplicity, stability of reagents, lack of radiation hazard and relatively inexpensive equipment. In the ELISA assay, antigen is fixed to a solid phase, incubated with test serum, then incubated with species-specified anti-immunoglobulin labeled with enzyme. Thus, enzyme adherent to the solid phase is related to the amount of antibody bound in the first incubation phase. Substrate is added and the enzyme activity is related to antibody concentration.

Sahu et al. (1979) developed an ELISA test for CEM. Soluble CEMO antigens, obtained from a sonicated saline suspension of CEMO, are fixed to U-bottom wells of microtiter plates. Anti-equine IgG is labeled with alkaline phosphatase and p-nitrophenylphosphate disodium is used as the indicator substrate. Positive serum samples have an optical density (OD) at 405 nm that is 2.5 times the average OD of controls. Samples positive at a 1:20 or higher dilution of serum are considered positive for CEM.

1.5. EPIDEMIOLOGICAL STUDIES

The purpose of epidemiological screening tests is to quickly and reliably separate the exposed population from the total test population. The suspect population is then subjected to additional specific testing. Since the function of a screening test is to eliminate only unexposed subjects, the tests used should have a low probability of false negative results, while a certain percentage of false positive results are quite acceptable.

Serological tests can be excellent epidemiological screening tests. The test samples are easily obtained and stable at -20°C for prolonged storage. Serological tests for bacterial infections are easily standardized, relatively economical, and less labor intensive than bacteriological culture techniques. Some characteristics of serological tests, such as retention of seropositivity after resolution of infection, allow a better estimation of total population exposure to an agent, since bacteriological methods give only a presently infected population estimate.

1.5.A. Equine Studies

Initial investigations of serological methods for CEM detection in horses found low-titered antibody levels against the CEMO in the presumedly unexposed horse population. Low-titered, non-specific reactions are common in serological tests. These results may represent serological cross-reactions between antigens of commensal or environmental organisms and those of the CEMO.

Benson et al. (1978), using the SAT method, found titers of 20 and occasionally up to 40 in horses with no known contact with CEMO.

Croxton-Smith et al. (1979) found 38% of their unexposed horse population had C-FIX test reactions at the 1:2 serum dilution and an additional 10% reacted at the 1:4 serum dilution. Fernie et al. (1979) used serum samples from two groups of unexposed horses. One group of frozen serum samples was obtained in 1976, prior to the first outbreak of CEM in England. The second group of serum samples was taken from clinically healthy horses in 1977 and 1978. Using the PHA for CEM, they found only 2.3% of the samples from 1976 had titers greater than 8, with the highest titer being 32. The PHA results for the 1977 and 1978 samples showed that 40% had titers greater than 8, with the highest being 64. Similar results were found in serum samples taken from clinically healthy Thoroughbred mares from Kentucky during the 1978 CEM outbreak in the United States. Of these samples, 22% had PHA titers greater than 8, with the highest titer being 128 (Fernie et al. 1979).

Croxton-Smith et al. (1978) found that the incidence of low-titered results increased with the age of the horse. Fernie et al. (1979) proposed that the increasing background titers found in clinically healthy mares may be due to indirect exposure to CEMO and subsequent sub-clinical infections. The increased background titers against CEMO between the 1976 and 1977-78 clinically healthy horse populations correlates with the increased opportunity for CEMO exposure.

1.5.B. Human Studies

Taylor and Rosenthal (1978a, 1978b, and Taylor 1979), using the SAT method of Benson et al. (1978), found a higher percentage of agglutinins to CEMO in patients attending a genito-medical clinic than in

other test populations. The percentage of patients with agglutinins to CEMO, with titers greater than or equal to 20, varied from 2% among women attending an antenatal clinic to 22% among women attending a genito-medical clinic. There was a 7% rate among healthy adult females. Of men attending a genito-medical clinic, 13% were positive compared with only 4% of healthy males. Additional studies performed on men with non-gonococcal urethritis found agglutinins to CEMO, with titers greater than or equal to 20, in 37.6% of those tested; 12.5% showed a four-fold or greater rise in titer during the course of their illness. These observations suggest that the CEMO or related organism may be involved in human venereal disease. Attempts to isolate CEMO from these patients have been unsuccessful.

1.5.C. Bovine Studies

Corbel and Brewer (1980), using the SAT method of Benson et al. (1978), found agglutinins to CEMO in a high percentage of bovine samples from the United Kingdom. However, 96.2% had titers less than or equal to 40, with 69.7% having titers less than or equal to 20. The significance of these results is not known, and they may simply represent a higher normal background titer to CEMO in cattle.

1.6. PROPOSED STUDY

The primary objective of this study is to identify species with a high probability of CEMO exposure. Another objective is to compare the serological methods for CEM detection, in terms of both test results and technical aspects of test mechanics. The final objective is to examine the morphology of the streptomycin sensitive strain of CEMO.

Species to be tested include sheep, horses, dogs, cattle, pigs and humans. Three serological tests for CEM detection will be performed on 200 serum samples from each test group. An additional 400 human samples will be tested to give three human population groups similar to those of Taylor and Rosenthal (1979a).

The serological tests for CEM used in this study will be:

1. The PAT method of Swerczek (1979), using the more sensitive non-Thoroughbred procedure.
2. A C-FIX method based on the work of Bryans et al. (1980).
3. The PHA method of Fernie et al. (1979).

All three tests will be performed on all serum samples if sample volume is sufficient. Samples will be screened at an initial dilution. All reactive samples at the screening dilution will be further titrated to determine final serum titer.

The streptomycin sensitive strain of CEMO will be examined using scanning and transmission electron microscopy techniques. It is known that the streptomycin resistant strain of CEMO is encapsulated (Swaney and Breese 1980), and that the capsule is responsible for some of the CEMO antigenic specificity (Bryans et al. 1980). The goal of this portion of the study is to determine if the streptomycin sensitive strain is encapsulated and, if so, how that capsule compares to that of the streptomycin resistant strain.

It is hoped that the results of this preliminary work will be useful as a guide for further research on the habitat and host-range of the CEMO.

CHAPTER II

METHODS AND MATERIALS

This chapter describes in detail the various components and procedures for the serological tests performed in this study. It also briefly covers the factors involved in the electron microscopy studies. Section 1 covers the obtaining and storage of serum samples for the epidemiological study. The next section deals with growth and maintenance of the two strains of CEMO. Actual procedures for the serological tests, including reagent preparation and necessary preliminary test procedures, are covered in section 3. The final section deals with the preparation of the CEMO for electron microscopy studies.

2.1. SERUM SAMPLES

Serum samples were obtained from the Animal Health Diagnostic Laboratory at Michigan State University, the Michigan Department of Public Health, the Michigan Department of Agriculture (Giegley Laboratory), the Pathology Department of Michigan State University, a local slaughter house and several area horse farms. All samples were drawn in 1979 and stored at -20°C. Minimum acceptable sample volume was set at one ml of serum. Grossly lipemic or hemolyzed samples were rejected.

At least two-hundred samples were collected from each animal group. The criteria used in sample selection were based on the known distribution of CEM antibodies among horses. Samples known to be from young or

neutered animals were rejected since the disease is sexually transmitted in horses. Samples known to be from male animals were rejected to avoid the problem of serological non-reactivity found in equine passive carriers of CEMO. Thus samples selected were primarily from sexually mature, unaltered females.

Over six-hundred serum samples from both men and women were collected from the Michigan Department of Public Health. Samples were selected from those submitted for VDRL (syphilis) or rubella testing. Patient confidentiality was preserved by recording only the age and sex of the donor. These samples were divided into 3 groups of approximately two-hundred each. Groups were selected to facilitate comparison to a preliminary study conducted in England by Taylor et al. (1978). Rubella samples selected were primarily from women of child-bearing age. This group was chosen to correspond to Taylor's antenatal group. The VDRL samples selected were divided into 2 groups based on the reason given for test submission. Pre-employment or pre-marital VDRL screening samples were considered representative of the normal population, while samples submitted for syphilis diagnosis or treatment were considered a high risk group, in the sense that they are more likely than the general population to have been exposed to venereal disease. The screening population and the high risk group correspond to Taylor's healthy and VD patient group.

2.2. CONTAGIOUS EQUINE METRITIS ORGANISM

Two strains of CEMO were obtained, under USDA permit #8641, from Dr. Thomas Swerczek (University of Kentucky, Lexington, KY 40546). One strain, #78-188, was a streptomycin resistant strain and the other,

#79-314, was a streptomycin sensitive strain. The streptomycin resistant CEMO is considered the type culture, and as such was used in the C-FIX and PHA antigen preparations. The streptomycin sensitive CEMO was examined with electron microscopic techniques to compare to previous studies of the streptomycin resistant CEMO.

Both strains were maintained on chocolate agar plates under microaerophilic conditions at 37°C. Plates were made with 10% sterile horse blood in Columbia agar base.[1] Microaerophilic conditions of 5-10% CO_2 in air were obtained with a CO_2 generating system (GasPak)[1] without using a palladium catalyst.

2.3. SEROLOGICAL TESTS

2.3.A. Plate Agglutination Test (PAT)

i. Reagents. All reagents for the PAT were supplied by Dr. T. Swerczek. The PAT antigen consisted of a stable suspension of intact CEMO. A PAT buffer was used to dilute samples to determine final titer.

ii. Procedure. In the screening procedure, 0.060 ml of serum was mixed with 0.025 ml of PAT antigen on a glass plate. Results were read after a 10 minute room temperature incubation. Results were graded 0 to 4+, with 0 indicating no visible agglutination and 4+ consisting of flocculent agglutination, yielding a ring pattern with clearing of the test area. Plates were covered during incubation to prevent evaporation. Test results were read using a diffuse light source beneath the test plate.

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[1] BBL, Div. of Becton, Dickinson and Co., Cockeysville, MD.

All samples with a result of 3+ or greater in the screening test were serially diluted in PAT buffer, heat inactivated at 56°C for 45 minutes and retested to determine final serum titer.

2.3.B. Complement Fixation (C-FIX)

i. Reagents. The C-FIX reagents and CEMO antigen were prepared according to the method of Bryans et al. (1979). The antigen was prepared from the streptomycin resistant strain of CEMO, #78-188. For more information on C-FIX antigen preparation see Appendix A. A tris buffered C-FIX diluent, pH 7.4 ± .04 was used for all reagent and serum dilutions. Sheep RBCs, anti-sheep serum (hemolysin) and lyophilized guinea pig complement were obtained from commercial sources.[1] All test and preliminary titrations were performed using Cooke Microtiter[2] U-bottom plates, microdiluters and pipette droppers.

ii. Procedures. Preliminary testing of diluent, hemolysin, complement and antigen were performed according to the procedures in Delaat (1974). Checkerboard titrations of hemolysin and complement were used to determine the optimal working concentration of both reagents. The concentration of complement used in the test procedure was 2.5 times that which caused 50% hemolysis of an equal volume of 1% sensitized sheep RBC suspension (2.5 HD_{50}). Hemolytic and anti-complementary (AC) activity of the antigen was checked according to the procedure in Delaat (1974). Antigen reactivity was matched in a C-FIX antigen supplied through Dr. J. Bryans.

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[1]Colorado Serum Co., Denver, CO.

[2]Dynatech Laboratories, Inc., Alexandria, VA.

The test procedure followed was a standardized micromethod that used 0.025 ml volumes for all reagents (Delaat 1974). To perform the test, samples were diluted in C-FIX diluent and heat inactivated at 56°C for 30 minutes immediately prior to testing. Equal volumes of diluted serum, antigen and complement (2.5 HD_{50}) were placed in each test well. Plates were covered, mixed and incubated for 2 hours at room temperature. Following incubation, an equal volume of a 1% sensitized sheep RBC suspension was added to all wells. Plates were then incubated for 30 minutes at 37°C, mixing after 15 minutes and again at 30 minutes. Test results were recorded after the cells had settled. Serum titer is defined as the highest serum dilution to yield a 2+ or greater result, which is hemolysis of less the 50% of the sensitized sheep RBCs.

Test controls included in each run were antigen hemolytic and AC controls, 50% and 100% lysis complement controls, diluent hemolytic controls, known positive and negative samples, and individual AC controls. Anticomplementary samples do not yield valid test results and were recorded simply as AC.

Preliminary titrations were performed on approximately 15 samples from each animal group and the screening human group to determine the test screening dilution for each group. These presumed unexposed samples were selected from those which had no reaction with the PAT. Samples were diluted 1:4 in C-FIX diluent and heat inactivated. Using the microtiter equipment, additional 2-fold serial dilutions were made so that serum dilutions of 1:4 through 1:256 were tested. Since no preliminary samples were reactive at the initial 1:4 dilution, all samples were screened at 1:4. Any samples with test results of 2+ or greater at this

screening dilution were serially diluted and retested to determine final serum titer.

2.3.C. Passive Hemagglutination Test (PHA)

i. Reagents. The CEMO antigen preparation contained soluble antigens obtained from a sonicated CEMO suspension, strain #78-188. Cellular debris and intact bacteria were removed through ultracentrifugation. The antigen (supernatant) was stored at -20°C.

Citrated blood (ACD) was collected from several 15 week-old turkeys. These cells were washed in saline and the buffy coat removed. Washed RBCs were subsequently preserved with formaldehyde using the method of Sequeira and Eldridge (1973). Portions of the formolized RBCs were resuspended in a phosphate buffered saline (PBS), pH 7.2, to a 4% suspension and treated with a 0.0005% w/v tannic acid solution. Tanned RBCs were sensitized with CEMO antigen by mixing 1 volume of 4% tanned RBCs with 4 volumes of PBS (pH 6.4) and 1 volume of CEMO antigen. The mixture was incubated at 37°C for 30 minutes, washed several times in saline, then reconstituted to a 1% suspension with 1% bovine serum albumin (BSA) in saline. For more information on PHA antigen preparation, including details on turkey RBC treatment, see Appendix A.

All tests and preliminary titrations were performed using Cooke Microtiter U-bottom plates, microdiluters and pipette droppers.

ii. Procedures. The concentration of CEMO antigen used to sensitize the tanned RBCs was determined through preliminary titrations. The selected antigen dilution yielded a clear cell sedimentation pattern with both positive and negative sera, and antigen reactivity was matched to a

PHA antigen prepared at Wellcome Laboratories and supplied through Dr. D. S. Fernie.

Samples were heat inactivated for 30 minutes at 56°C, diluted in 1% BSA saline and adsorbed with an equal volume of packed formolized RBCs. Test wells contained 0.025 ml of diluted serum and 0.050 ml of antigen. Individual serum controls contained 0.025 ml of diluted serum with 0.050 ml of 1% formolized RBCs in 1% BSA saline. Additional controls included in each test run were known positive and negative serum samples and diluent controls. Plates were covered, mixed by swirling and incubated for 2 hours at room temperature. A negative sedimentation pattern was defined as a tight button of cells whose diameter was less than 1/2 the diameter of the bottom of the reaction well.

Preliminary titrations were performed on approximately 20 samples from each animal group and the screening human group to determine the test screening dilution for each group. These presumed unexposed samples were selected from those that were non-reactive with the PAT and negative (excluding AC) with C-FIX. Preliminary titrations were performed in duplicate using both adsorbed and unadsorbed sera. Samples were initially diluted 1:8 and additional 2-fold serial dilutions were made with the microtiter equipment. Serum dilutions of 1:8 through 1:128 were tested. Test results were coded 0 to 5 with 0 representing a negative result at the 1:8 dilution and 5 being a positive result at the 1:128 dilution. The screening dilution for each species was set at the dilution at or above the group's average coded PHA result plus 1 SD. Mass screening was performed on unadsorbed serum samples only if no significant differences were found between the preliminary titrations on adsorbed and

unadsorbed sera. All samples positive at the screening dilution were adsorbed with packed formolized turkey RBCs, serially diluted and retested to determine final serum titer.

2.4. ELECTRON MICROSCOPY STUDIES

2.4.A. Scanning Electron Microscopy (SEM)

The streptomycin sensitive strain of CEMO, #79-314, was grown in Schaedler broth[1] at 37°C under microaerophilic conditions. A 5 day-old culture was fixed in 1% gluteraldehyde overnight at 4°C, dehydrated with a graded alcohol series and placed on glass coverslips. Samples were sputter coated with gold and examined in an ISI Super III.[2]

2.4.B. Transmission Electron Microscopy (TEM)

The streptomycin sensitive strain of CEMO, #79-314, was grown in Schaedler broth[1] at 37°C under microaerophilic conditions. A 5 day-old culture was fixed with Karnovsky's solution, washed in Zetterqvist buffer and post-fixed in a Zetterqvist-osmium solution. The sample was dehydrated with a graded alcohol series, using propylene oxide in the final dehydration phase. The CEMO was embedded in a Epon-Araldite resin mixture. Ultra-thin sections were stained with uranyl acetate and lead citrate solutions and examined on a Philips EM-201.[3]

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[1] BBL, Div. of Becton, Dickinson and Co., Cockeysville, MD.

[2] International Scientific Instruments, Inc., Santa Clara, CA.

[3] N. V. Philips, Gloeilampenfabrieken, Eindhoven, Netherlands.

CHAPTER III

RESULTS AND DISCUSSION

3.1. INTRODUCTION

The primary objective of this study is the identification of species with a high probability of CEMO exposure. Secondary objectives include a comparison of test methods in terms of test results and the technical aspects of test mechanics, and a morphological study of the streptomycin sensitive strain of CEMO.

Presumed exposure to CEMO was estimated from the strength and distribution of titered test results in species with no defined normal group. Human results were compared to the presumed normal population to discern whether there was CEMO exposure.

Contingency tests were used to check for correlation of test results. Only results from samples tested by all three test methods were used to generate the contingency tables for correlation testing.

Test methods were also compared on a technical basis. Points of comparison included serum requirements, reagent preparation and stability, technical skills required for test performance, and suitability for use as an epidemiological screening test.

The morphological study of the streptomycin sensitive strain of CEMO included SEM examination of shape and surface detail, and TEM studies of internal structures. The EM work was compared to previous

studies performed on the streptomycin resistant strain of CEMO by Swaney and Breese (1980) and Sahu and Dardiri (1981).

A description of the figures and tables of results is in section 3.2. The next section evaluates the serological test results concerning the possibility of CEMO exposure. Correlation of serological test results is examined in section 3.4. The final section consists of the morphological study of the streptomycin sensitive strain of CEMO.

3.2. DESCRIPTION OF FIGURES AND TABLES

3.2.A. Percentage Positive to CEMO

Table 1 lists the percentage positive (reactive) to CEMO antigens by test method and species, positive referring to samples reactive at or above the screening dilution. The PAT used straight serum for screening, while the serum dilutions used for C-FIX and PHA screening were determined on an individual species basis through preliminary titrations of selected samples from each group.

With the C-Fix, no preliminary titrated samples from any group were positive at the initial dilution of 1:4. Therefore, all groups were screened at a 1:4 dilution of serum. The titered PHA results varied among the species. Human, sheep, dog and swine samples were screened at a 1:8 serum dilution. Horse and cattle samples had measurable PHA titers, and thus they were screened at a 1:16 and 1:32 serum dilution respectively. For preliminary PHA titration results, see Appendix B.

3.2.B. Titered Results

Samples reactive at the screening dilution were serially diluted to determine final serum titer. No samples were reactive beyond the

TABLE 1. SEROLOGICAL TEST RESULTS, PERCENTAGE POSITIVE* TO CEMO ANTIGEN.

	PAT			C-FIX					PHA		
GROUP TESTED	TOTAL	POS	(%)	TOTAL	POS	(%)	AC	(%)	TOTAL	POS	(%)
HUMANS											
SCREENING	209	0	(0.0)	201	10	(5.0)	35	(17.4)	205	5	(2.4)
RUBELLA	206	0	(0.0)	200	8	(4.0)	20	(10.0)	206	3	(1.5)
HIGH RISK	249	0	(0.0)	200	20	(10.0)	30	(15.0)	204	16	(7.8)
SHEEP	205	5	(2.4)	196	3	(1.5)	50	(25.6)	198	32	(16.2)
DOG	249	4	(1.6)	201	0	(0.0)	135	(67.2)	205	12	(5.9)
PIG	222	0	(0.0)	209	0	(0.0)	45	(21.5)	210	30	(14.3)
HORSE	258	8	(3.1)	200	2	(1.0)	20	(10.0)	201	32	(15.0)
CATTLE	244	24	(9.8)	234	9	(3.8)	54	(23.0)	240	25	(10.4)

* POSITIVE REFERS TO SAMPLES REACTIVE AT OR ABOVE THE SCREENING DILUTION.

screening dilution with PAT. The C-FIX and PHA titers are presented in Tables 2 and 3 respectively. Data in these tables include the results from all the samples tested by each of the methods.

3.2.C. Contingency Tables

Table 4 compares the test results among the methods for all samples and for each separate test group. The data in Table 4 include results only from those samples which were tested by all three serological methods. Each block of Table 4 gives the number of samples per test group with each of the twelve possible combinations of C-FIX, PAT and PHA results. The column on the right and the lower two rows of each block contain the total number of positive (reactive) or negative results for each test method. The last entry in the right column of each block is the total number of samples tested from that group. For example, in the all species block, 46 samples were C-FIX positive, PAT negative and PHA negative; 110 were C-FIX negative, PAT negative and PHA positive; 351 were anticomplementary, PAT negative and PHA negative; and a total of 1637 samples were tested.

3.2.D. Electron Microscopy Studies

Figures 2 through 6 are electron photomicrographs of the streptomycin sensitive strain of CEMO. The scanning EM photomicrographs are presented on pages 55 and 56. The transmission EM photomicrographs are on page 57. Labelled features of the CEMO include: Capsule (C), outer membrane (O), dense intermediate layer (D), and cytoplasmic membrane (P). All ultrathin sections were stained with uranyl acetate and lead citrate.

TABLE 2. DISTRIBUTION OF COMPLEMENT FIXATION TITERS FOR CEMO ANTIGENS.

GROUP TESTED	AC	C-FIX TITERS NEG	4	8	16	32	64	TOTAL
HUMANS ALL GROUPS	85	478	7	16	14	1	-	601
SCREENING	35	156	3	3	3	1	-	201
RUBELLA	20	172	-	6	2	-	-	200
HIGH RISK	30	150	4	7	9	-	-	200
SHEEP	50	143	1	-	2	-	-	196
DOG	135	66	-	-	-	-	-	201
PIG	45	164	-	-	-	-	-	209
HORSE	20	178	-	2	-	-	-	200
CATTLE	54	171	-	5	4	-	-	234

AC - ANTI-COMPLEMENTARY

TABLE 3. DISTRIBUTION OF PASSIVE HEMAGGLUTINATION TITERS FOR CEMO ANTIGENS.

GROUP TESTED	PASSIVE HEMAGGLUTINATION TITERS NEG*	8	16	32	64	128	256	512	1024	TOTAL
HUMANS ALL GROUPS	591	10	10	4	-	-	-	-	-	615
SCREENING	200	1	3	1	-	-	-	-	-	205
RUBELLA	203	-	1	2	-	-	-	-	-	206
HIGH RISK	188	9	6	1	-	-	-	-	-	204
SHEEP	166	-	-	5	19	3	3	2	-	198
DOG	193	6	6	-	-	-	-	-	-	205
PIG	180	16	2	1	1	5	3	-	2	210
HORSE	169	NT	15	10	2	2	3	-	-	201
CATTLE	215	NT	NT	11	8	4	1	1	-	240

* NEGATIVE AT INITIAL SCREENING DILUTION. NT - NOT TESTED.
HORSE SERA SCREENED AT 1:16 DILUTION, CATTLE SERA SCREENED AT 1:32 DILUTION OF SERA, ALL OTHER GROUPS SCREENED AT 1:8 DILUTION OF SERA.

TABLE 4. CONTINGENCY TABLES FOR COMPARISON OF SEROLOGICAL TEST RESULTS.

	C-FIX	+		−		AC		PHA/ GRAND TOTALS
	PAT	+	−	+	−	+	−	
CUMULATIVE ALL SPECIES	PHA +	1	2	7	110	1	29	+ 150
	PHA −	3	46	21	1059	7	351	− 1487
C-FIX TOTALS		+ 52		− 1197		AC 388		1637
PAT TOTALS		+ 40		− 1597				

HUMANS ALL GROUPS	PHA +	0	1	0	20	0	3	+ 24
	PHA −	0	37	0	455	0	81	− 573
C-FIX TOTALS		+ 38		− 475		AC 84		597
PAT TOTALS		+ 0		− 597				

HUMANS SCREENING	PHA +	0	1	0	4	0	0	+ 5
	PHA −	0	9	0	147	0	35	− 191
C-FIX TOTALS		+ 9		− 151		AC 35		196
PAT TOTALS		+ 0		− 196				

HUMANS RUBELLA	PHA +	0	0	0	2	0	1	+ 3
	PHA −	0	8	0	171	0	19	− 198
C-FIX TOTALS		+ 8		− 173		AC 20		201
PAT TOTALS		+ 0		− 201				

HUMANS HIGH RISK	PHA +	0	0	0	14	0	2	+ 16
	PHA −	0	20	0	136	0	28	− 184
C-FIX TOTALS		+ 20		− 150		AC 30		200
PAT TOTALS		+ 0		− 200				

	C-FIX	+		−		AC		PHA/ GRAND TOTALS
	PAT	+	−	+	−	+	−	
SHEEP	PHA +	0	1	0	22	0	7	+ 30
	PHA −	0	2	4	117	1	42	− 166
C-FIX TOTALS		+ 3		− 143		AC 50		196
PAT TOTALS		+ 5		− 191				

DOG	PHA +	0	0	0	4	0	6	+ 10
	PHA −	0	0	1	61	3	126	− 191
C-FIX TOTALS		+ 0		− 66		AC 135		201
PAT TOTALS		+ 4		− 197				

PIG	PHA +	0	0	0	22	0	8	+ 30
	PHA −	0	0	0	142	0	37	− 179
C-FIX TOTALS		+ 0		− 164		AC 45		209
PAT TOTALS		+ 0		− 209				

HORSE	PHA +	0	0	3	27	0	2	+ 32
	PHA −	0	2	2	146	0	18	− 168
C-FIX TOTALS		+ 2		− 178		AC 20		200
PAT TOTALS		+ 5		− 195				

CATTLE	PHA +	1	0	4	15	1	3	+ 24
	PHA −	3	5	14	138	3	47	− 210
C-FIX TOTALS		+ 9		− 171		AC 54		234
PAT TOTALS		+ 26		− 208				

3.3. EVALUATION OF CEMO EXPOSURE

3.3.A. Animal Groups

The animal groups were evaluated as to their probability of CEMO exposure based on the strength and distribution of titered results. The screening dilution used for each group was selected to eliminate at least 66% of each group from further titration. Thus, titered results at or near the screening dilution were assumed to represent the tail of the distribution of non-significant serum titers. Background CEMO titers have been demonstrated in horses and cattle with SAT (Benson et al. (1978, Croxton-Smith et al. 1978, Fernie et al. 1979, Corbel and Brewer 1980) and may be due to cross-reacting antibodies to antigens of similar organisms.

Using the above stated criteria, sheep and pigs appear to have a high probability of CEMO exposure. Their PHA titered results appear to have a bimodal distribution. This is highly suggestive of the existence of two populations within each of these two species which differ in their reactivity to CEMO antigens (see Table 3).

Sheep PHA results are clearly bimodal, with approximately 85% of the samples non-reactive at the 1:8 dilution and the titered reactive samples clustered around 64. No samples had titers of 8 or 16, yielding a clear and definite division between reactive and non-reactive samples.

The bimodal distribution of the swine samples is not as well defined as that of the sheep. Samples were screened at a 1:8 dilution and approximately 85% were non-reactive. Titered results range from 8 to 1024. The frequencies of the low-titered results, 8 and 16, suggest they

are in the tail of the distribution curve of the predominantly non-reactive samples. The higher titered results cluster around 128 and the distribution patterns of the two populations probably overlap in the titer range of 32 to 64.

The remaining animal groups, dogs, horses, and cattle, do not present evidence of CEMO exposure through titered test results (see Tables 1, 2 and 3). Dogs have few reactive samples (Table 1) and titered results are at or near the screening dilution used for each test (Tables 2 and 3). A low percentage of horse samples are reactive to CEMO with PAT or C-FIX (Table 1). While 15% of the horse samples are PHA reactive, the frequencies of titered results are consistent with those expected in the tail of the distribution curve of predominantly non-reactive samples. Cattle PHA results show this same pattern of decreasing frequencies of higher titered results (Table 3). The percentage of PAT reactive samples is much higher for cattle than any other group (Table 1), however, no sample was reactive beyond the screening dilution. Cattle had the highest percentage of C-FIX reactive samples of all the animal groups (Table 1) and the titered results are tightly clustered (Table 2). However, overall the number of reactive samples is too low to cite as evidence of CEMO exposure in cattle.

Previous epidemiological studies have been performed on horses and cattle (see sections 1.5.A. and 1.5.C.). Fernie et al. (1979) tested presumably unexposed Kentucky Thoroughbred mares and found approximately 78% had PHA titers of 8 or less with the highest titer reaching 128. The results of this present study, which found 85% of the horse samples had PHA titers of 8 or less with the highest titer reaching 256, are

comparable to the previous work of Fernie et al. (1979). Diagnostic PHA titers in horses were set by Fernie et al. (1979) at titers equal to or greater than 256. However, the commonly accepted error range for serological tests is plus or minus one 2-fold dilution, therefore, no special significance can be placed on those few samples in the present study with titers of 256. The epidemiological study of cattle performed by Corbel and Brewer (1980) found background titers to CEMO ranged slightly higher than those found in horses. The results of this present study supports their findings.

3.3.B. Human Studies

The existence of a defined normal group, to use as a reference, allows for a more detailed analysis of serological test results. The three human groups were compared first on the basis of percentage reactive by any method. The 2x2 contingency test used to examine the null hypothesis of no difference between human groups is presented in Table 5. Contingency tests can be used to compare distribution of test results. The distribution of the defined normal group is used to generate the expected frequencies for the test results, under the null hypothesis of no difference between groups. The deviation of the observed frequencies from the expected frequencies is used to calculate chi-squared (χ^2). In the case of 2x2 contingency tests, the χ^2 was calculated with the Yates correction for continuity (Tate and Clelland 1957):

$$\chi^2 = \Sigma(|f_{exp} - f_{obs}| - 1/2)^2 / f_{exp}.$$

Table 5. Comparison of human groups, all methods.

	Reactive		Non-reactive		Totals	
Group	Number	χ_1^2	Number	χ_2^2	Number	χ^2
Screening	15		194		209	
Rubella	11	0.730	195	0.096	206	0.826
High Risk	35	19.420	213	1.345	249	20.765

The rubella group's χ^2 value of 0.826 with 1 degree of freedom (df=1) allows rejection of the null hypothesis of no difference between groups only at the 46.3% level of confidence (Table 5), thus the null hypothesis must be accepted. However, the high risk group deviates significantly from the screening group. The high risk χ^2 value of 20.765 with df=1 allows rejection of the null hypothesis at the 99.9% level of confidence (Table 5). The major contribution of this highly significant χ^2 was that of the much higher than expected number of reactive samples in the high risk group.

This highly significant finding using results from all test methods allows further analysis of the high risk group on an individual test method basis. Again, in testing the null hypothesis of no difference between human groups, the screening group's results were used to generate expected frequencies.

Table 6. Comparison of human groups, C-FIX test method.

	Reactive		Non-reactive		Totals	
Group	Number	χ^2_1	Number	χ^2_2	Number	χ^2
Screening	10		191		201	
High Risk	20	11.185	180	0.480	200	11.665

Table 7. Comparison of human groups, PHA test method.

	Reactive		Non-reactive		Totals	
Group	Number	χ^2_1	Number	χ^2_2	Number	χ^2
Screening	5		200		205	
High Risk	16	22.261	188	0.557	204	22.818

The comparison of high risk and screening groups on individual test method results found that the high risk group deviated significantly from the screening group with both the C-FIX and PHA test methods. The PAT method was not evaluated since no human samples reacted with that method. Both the high risk group's C-FIX χ^2 of 11.665 and PHA χ^2 of 22.818, with df=1, allowed rejection of the null hypothesis of no difference between human groups at the 99.9% confidence level (Tables 6 and 7). In both test methods, the major contribution to the highly significant chi-squared value was the much higher than expected number of reactive samples in the high risk group.

The human groups in this present study were selected to compare to previous human epidemiological studies performed by Taylor and Rosenthal (1978a) in England. Taylor and Rosenthal divided their population into 3 groups: healthy adults, venereal disease (VD) patients and antenatal patients. The present study's corresponding groups were screening, high risk, and rubella groups. Taylor and Rosenthal tested their samples as a 1:20 serum dilution with the SAT of Benson et al. (1978). They found reaction samples in 5.5% of the healthy adults, 17.4% of the VD patients and 2.0% of the antenatal group. Using the PHA and C-FIX results from this present study, reactive samples were found in 7.3% of the screening group, 17.6% of the high risk group, and 5.3% of the rubella group. The finding of a highly significant statistical difference between the high risk group versus the other human groups strongly supports the hypothesis of Taylor and Rosenthal that the CEMO or related organism may be involved in human venereal disease.

3.4. CORRELATION OF SEROLOGICAL TEST RESULTS

The serological tests were first evaluated for correlation of results on a cumulative basis, independent of sample source. Table 8, the contingency table used to test correlation of results was generated from the data in Table 4. Contingency tests can estimate the independence of distinct characteristics within a defined group. Under the null hypothesis of independence, the expected frequency of samples with any combination of distinct characteristics equals the product of the probability of occurrence for each characteristic alone times the total number of samples. The deviations of the observed frequency from the

TABLE 8: CHI-SQUARED VALUES FOR TESTING INDEPENDENCE OF SEROLOGICAL TEST METHODS.

			ALL SPECIES			CATTLE			HORSE		
C-FIX	PHA	PAT	F_{EXP}	F_{OBS}	x^2	F_{EXP}	F_{OBS}	x^2	F_{EXP}	F_{OBS}	x^2
–	–	–	1060.8	1059	0.003	136.4	138	0.019	145.8	146	0.000
–	–	+	26.6	21	1.179	17.1	14	0.562	3.7	2	0.781
–	+	–	107.0	110	0.084	15.6	15	0.023	27.8	27	0.023
–	+	+	2.7	7	6.848	2.0	4	2.000	0.7*	3	
AC	–	–	343.8	351	0.151	43.1	47	0.353	16.4	18	0.156
AC	–	+	8.6	7	0.298	5.4	3	1.067	0.4*	0	
AC	+	–	34.7	29	0.936	4.9	3	0.737	3.1	2	0.390
AC	+	+	0.9*	1		0.6*	1		0.1*	0	
+	–	–	46.1	46	0.000	7.2	5	0.672	1.6*	2	
+	–	+	1.2*	3		0.9*	3		0.0*	0	
+	+	–	4.7	2	1.551	0.8*	0		0.3*	0	
+	+	+	0.1*	1		0.1*	1		0.0*	0	
POOL TOTAL			2.2	5	3.563	2.4	5	2.817	3.1	5	1.165
GRAND TOTAL					14.613			8.250			2.515

* FREQUENCIES POOLED PRIOR TO x^2 CALCULATION.

TABLE 8 (CONT'D.).

			Sheep			Dog			Pig		
C-FIX	PHA	PAT	F_{EXP}	F_{OBS}	x^2	F_{EXP}	F_{OBS}	x^2	F_{EXP}	F_{OBS}	x^2
–	–	–	118.0	117	0.008	61.5	61	0.004	140.5	142	0.016
–	–	+	1.1*	1		1.3*	1		0.0*	0	
–	+	–	21.3	22	0.023	3.2	4	0.200	23.5	22	0.096
–	+	+	0.6*	0		0.1*	0		0.0*	0	
AC	–	–	41.3	42	0.011	125.7	126	0.001	38.5	37	0.058
AC	–	+	2.5	2	0.100	2.6*	3		0.0*	0	
AC	+	–	7.5	7	0.033	6.6	6	0.055	6.5*	8	
AC	+	+	0.2*	0	0.261	0.1*	0		0.0*	0	
+	–	–	3.1	4		0.0*	0		0.0*	0	
+	–	+	0.1*	0		0.0*	0		0.0*	0	
+	+	–	0.5*	1		0.0*	0		0.0*	0	
+	+	+	0.6*	0		0.1*	0		0.0*	0	
POOL TOTAL			3.1	2	0.390	4.2	4	0.010	6.5	8	0.346
GRAND TOTAL					0.818			0.270			0.516

* FREQUENCIES POOLED PRIOR TO x^2 CALCULATION.

TABLE 8. (CONT'D).

			HUMAN-TOTAL			SCREENING			RUBELLA			HIGH RISK		
C-FIX	PHA	PAT	F_{EXP}	F_{OBS}	x^2	F_{EXP}	F_{OBS}	x^2	F_{EXP}	F_{OBS}	x^2	F_{EXP}	F_{OBS}	x^2
–	–	–	455.9	455	0.002	147.2	147	0.004	171.4	172	0.002	138.0	136	0.029
–	–	+	0.0*	0		0.0*	0		0.0*	0		0.0*	0	
–	+	–	19.1	20	0.042	3.9*	4		2.6*	2		12.0	14	0.033
–	+	+	0.0*	0		0.0*	0		0.0*	0		0.0*	0	
AC	–	–	80.6	81	0.002	34.1	35	0.024	18.7	18	0.026	27.6	28	0.006
AC	–	+	0.0*	0		0.0*	0		0.0*	0		0.0*	0	
AC	+	–	3.4*	3	0.047	0.9*	0		0.3*	1		2.4*	2	
AC	+	+	0.0*	0		0.0*	0		0.0*	0		0.0*	0	
+	–	–	36.5	37	0.007	9.7	9	0.051	7.9	8	0.013	18.4	20	0.139
+	–	+	0.0*	0		0.0*	0		0.0*	0		0.0*	0	
+	+	–	1.5*	1		0.3*	1		0.0*	0		1.6*	0	
+	+	+	0.0*	0		0.0*	0		0.0*	0		0.0*	0	
POOL TOTAL			4.9	4	0.165	5.1	5	0.020	3.0	3	0.000	4.0	2	1.000
GRAND TOTAL					0.218			0.099			0.041			1.478

* FREQUENCIES POOLED PRIOR TO x^2 CALCULATION.

expected frequency, under the hypothesis of independence, is a measure of correlation between characteristics. For Table 8, the expected frequencies were calculated using the marginal totals as estimates of the probability of occurrence of any individual test result. In cases where the expected frequency of a class was less than 2, class results were pooled to give an expected frequency greater than 2, prior to χ^2 determination (Tate and Clelland 1957). The null hypothesis tested was that of independence of test results.

The all-species (cumulative) chi-squared value of 14.613 at df=6 allowed rejection of the null hypothesis at the 97.5% level of confidence (Table 8). Further analysis on a population group basis found no correlation of test results in horses, sheep, dogs, pigs and humans. The χ^2 values for these groups were below 3.4, which allows rejection of the null hypothesis only at the 25% level of confidence, and thus the null hypothesis of independence must be accepted. The cattle chi-squared value of 8.25 allowed rejection of the null hypothesis only at the 78% confidence level.

Additional analysis of cattle test results, to determine correlation between any two test methods, was performed with pairwise contingency tables. However, because of low number of expected frequencies in some result categories, the exact probability of the observed and more extreme cases was used to evaluate correlation of test results (Tate and Clelland 1957). See Appendix C for details of this statistical evaluation.

Comparison of cattle test results, under the null hypothesis of independence, found no correlation in PHA and C-FIX results, but a highly significant correlation between PAT and C-FIX results, and PAT and PHA

results, at a greater than 95% level of confidence. Correlation of test results is of interest because the antigen sources for the serological tests were prepared differently, yielding different antigen combinations in each test antigen preparation, capsular antigens in the C-FIX and PAT methods and soluble antigens in the PHA method. The positive correlation of test results implies the existence of a group of cattle with antibodies to both combinations of CEMO antigens. This increases the probability that those antibodies were induced by CEMO exposure and are not due to cross-reacting antibodies.

The lack of correlation in serological test results in most species may be due to several factors such as differences in test antigen preparation, varying requirements of test conditions for the different species and the differential production of antibody types.

The PAT and C-FIX test antigen preparations consists of a suspension of intact CEMO while the PHA test antigen preparation contains soluble CEMO antigens. Thus the PAT and C-FIX tests were more sensitive to capsular antibodies while the PHA measured a wider range of antibodies to diverse CEMO antigens. The antigenicity of these various antigens may vary within the different species and affect their ability to react with the different test methods.

The three serological test methods were all prepared for use in horses, thus test conditions were not optimized for use in other species. In fact, the test procedures for the PAT varied within one species, with different antigen to serum ratios for Thoroughbred versus non-Thoroughbred horse breeds. The C-FIX method had the greatest number of problems due to the presence of interfering substances in some sera. Pig serum has been

known to contain substances which enhance guinea pig complement activity and cattle antibodies activate rabbit complement more readily than guinea pig complement (Tizard 1977), factors which limit the reliability of C-FIX test results in these species. A high percentage of dog samples were anticomplementary, which invalidates test results and seriously limits the usefulness of C-FIX testing in dogs. Any factors which interfere with one test method will also affect the correlation of test results.

The differential production of antibody types refers to both antibody class, IgM or IgG, and functional antibody type, such as agglutinating, precipitating or complement fixing antibody. On a molar basis, IgM antibodies have a much greater ability to form the visible reactions of agglutination, precipitation and complement fixation than IgG antibodies. Also the functional type of antibody can vary within the antibody class. Sahu et al. (1981) found that agglutinating antibodies to CEMO were the most consistent antibody type formed in experimentally infected ponies. By contrast, complement fixing antibodies were found only after the second experimental exposure to CEMO and then only in 40% of the ponies. Thus the overall reactivity of a serum sample is dependent on the number and length of CEMO exposure(s), the class and type of antibody produced, and the serological method used to detect CEMO antibodies.

3.5. COMPARISON OF TEST MECHANICS

This section deals with the practical aspects of the serological test methods such as serum requirements, reagent preparation and stability, technical skills required to perform test procedures and suitability for use as a serological screening test.

3.5.A. Plate Agglutination Test (PAT)

Plate agglutination tests are fast and easy to perform. Serum requirements are minimal, typically much less than 0.5 ml. Dr. T. Swerczek provided both the buffer and CEMO antigen for this project and the details of their preparation has not been published. The PAT antigen is a saline suspension of intact CEMO, diluted to a specific density with a simple buffered saline solution as the diluent. These reagents are stable for months at 0-4°C. The test procedure requires minimal technical ability: serum and antigen are mixed together on a glass plate and the resulting agglutination pattern is read after a 10 minute room temperature incubation period.

The main drawback of this method as a serological screening test is the lack of sensitivity. The short incubation period allows agglutination only by antibodies of high affinity and avidity, which can lead to false negative results. A second drawback to the PAT is the lack of a universal procedure. The two test procedures available, which vary in the ratio of serum to antigen, are specific for different breeds within one species. Thus it is questionable whether either procedure is suitable for use in other species.

3.5.B. Complement Fixation (C-FIX)

Complement fixation test methods can be adapted to micro techniques which utilize less than 0.5 ml of serum. As a class, C-FIX is a complex serological test requiring the careful balancing of several reagents. Most of these reagents can be obtained from commercial sources and stability may vary with different manufacturers. The C-FIX CEMO antigen takes 10 to 14 days to prepare, but is stable for months when refrigerated

(0-4°C). The tris buffer and hemolysin are also stable for months. However, the sheep RBCs and complement are both labile reagents, with sheep RBCs stable 21-28 days at 0-4°C and reconstituted complement for 21 days at -20°C. Complement activity is matched to the indicator system of hemolysin-coated sheep RBCs. Changes in any of these reagents requires retitration to determine optimal concentrations of each while keeping complement activity at 2.5 HD_{50} per test volume. Antigen and serum samples must be checked for their ability to non-specifically absorb complement (anticomplementary activity), which invalidates test results.

This 2-stage test has a total of approximately 4 hours of incubation periods. The serum samples are heat inactivated at 56°C for 30 minutes. The first (test) phase incubated at room temperature for 2 hours. After addition of the indicator system (coated sheep RBCs), a 30 minute incubation follows at 37°C. Results are read when the cell pattern has settled, which requires 30-60 minutes.

Two reagents, the hemolysin-coated sheep RBCs and working strength complement, must be prepared fresh prior to each test run. The complement is very labile and can be inactivated through improper handling.

In terms of test mechanics, the C-FIX test is the most complicated of the test methods evaluated. It requires more time and technical ability to perform than PAT or PHA test methods because of the need to balance labile and unstable reagents.

As a serological screening test, C-FIX has two major drawbacks. The first is the presence of anticomplementary activity in some stored serum samples. It is believed that protein aggregates form during serum

storage which are responsible for AC activity. Anticomplementary activity varied among the different species, ranging from 10% to over 67% of the samples tested. Since AC activity invalidates C-FIX results, it is unsuitable for use in species with a high percentage of AC activity.

The second drawback to C-FIX tests is the unpredictable effect of some species' serum on xenogenic complement. Swine serum has been known to enhance the activity of guinea pig complement, altering the careful balance of reagents. Some species' antibodies do not activate guinea pig complement well and thereby the C-FIX test would underestimate antibody levels. Both of these situations would yield high false negative results, a major drawback for an epidemiological screening test.

3.5.C. Passive Hemagglutination (PHA)

The PHA test is less complicated to perform than the C-FIX test method. Micro methods are available which use less than 0.5 ml of serum. The stock PHA reagents, formolized turkey RBCs, tannic acid solution, PBS and CEMO antigens are stable for months. However, the tanning of formolized cells and adsorption of CEMO antigens must be done immediately before use, a process taking approximately 1 hour.

The PHA is a one stage test in which adsorbed serum, diluent and antigen are added at one time, mixed and then read after 2 hours of room temperature incubation. The test plate must be protected from vibrations and sources of static electric charge, both of which could alter the antigen sedimentation pattern. The PHA is a reasonably easy test to perform once a working procedure has been established. However, problems in obtaining clear, reproducible sedimentation patterns can be very difficult to isolate. It took over 2 weeks to determine that the method used

to mix reagents and samples, gently swirling the test plates on the counter, was generating a static charge on the plates, causing an erratic sedimentation pattern.

The immunological basis of the PHA test is much less complex than that of the C-FIX test. In PHA, the binding of antigens by specific antibodies causes visible agglutination of the carrier erythrocytes. Complement fixation tests rely on the binding of antigens by specific antibodies to initiate the process of complement activation. Thus, in PHA the binding of antigen by antibody directly forms the visible end-point reaction,while in C-FIX, the reaction of antibody and antigen is just the first of a series of steps that result in the visible reaction.

The PHA is a suitable test for use as an epidemiological screening procedure. There are few interfering substances, the major one being anti-carrier antibodies. This problem is eliminated by adsorption of the serum with tanned but uncoated carrier cells before testing. The antigen preparation for PHA consists of soluble antigens, a more diverse group than the capsular antigens tested for by PAT and C-FIX. The sensitivity and simplicity of the PHA test make it suitable for use in many species without modification of test procedure.

3.6. ELECTRON MICROSCOPY STUDIES

Bacterial colonies of CEMO appear similar to Staphylococcal colonies; smooth, round, convex, glistening, cream-to-white colonies approximately 0.5 to 2.0 mm in diameter after 4 days growth. The streptomycin-sensitive CEMO grew at a slightly slower rate than the streptomycin-resistant strain on 10% clocolate Columbia agar. Colonies from both

strains glide easily on the media surface. The streptomycin-sensitive strain acted very sticky. When pulled from the media, it would stretch, then snap up to the loop or back to the media. The appearance and behavior of the streptomycin-sensitive strain suggests the presence of a capsule, which is known to exist in the streptomycin-resistant CEMO.

3.6.A. Scanning Electron Microscopy Studies

Previous scanning EM studies of the streptomycin-resistant strain found predominantly rod forms from media which supported good growth of CEMO and coccal forms from less supportive media (Swaney and Breese 1980, Sahu and Dardiri 1980). For this study, 5 day-old cultures of the streptomycin-sensitive CEMO grown in Schaedler broth media were examined. Coccal forms predominated, most often in masses as in Figure 2. Long strands of pleomorphic rods, many ending in blunt, club-like forms, were also found (Figure 3). These strands were often associated with the masses of CEMO. Some of these strands of rod forms terminated in coccal forms as in Figure 4. Very few organisms were found alone and this strong cell adherence suggests the presence of a capsule. No signs of pili or flagella were noted in the SEM photomicrographs.

The finding of both rod and coccal forms in one broth culture suggests that the CEMO shape is dependent on the growth phase when viewed. The CEMO is rod-shaped during the exponential growth phase where conditions favor bacterial growth, and coccal-shaped when growth conditions are minimal. This type of growth-phase dependent morphology is known to occur in other bacterial groups. An example is Acinetobacter of the Neisseriaceae family (Baumann et al. (1968a). This pattern of

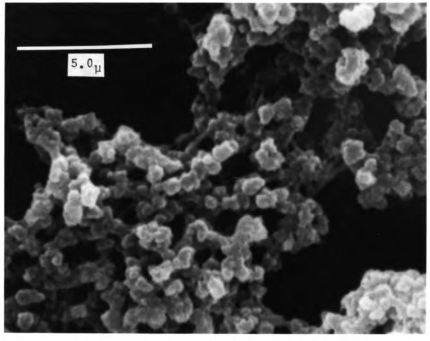

Figure 2. SEM photomicrograph of streptomycin-sensitive CEMO at 7,100X showing a mass of coccoid-shaped CEMO.

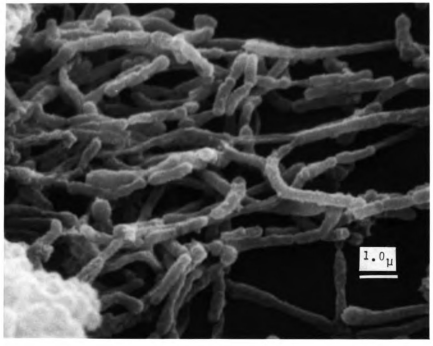

Figure 3. SEM photomicrograph of streptomycin-sensitive CEMO at 10,000X demonstrating pleomorphic rod-shaped CEMO.

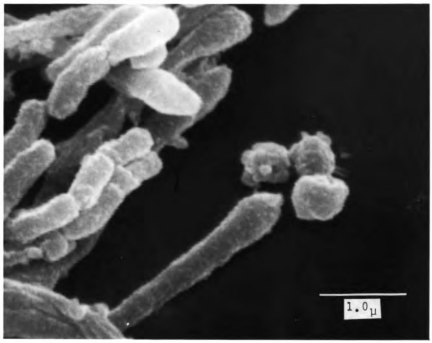

Figure 4. SEM photomicrograph of streptomycin-sensitive CEMO at 23,000X demonstrating rod forms terminating in coccoid-shaped organisms.

growth-phase dependent morphology makes taxonomic classification based on shape highly questionable.

The transmission EM photomicrographs of the streptomycin-sensitive strain of CEMO, Figures 5 and 6, demonstrate a thin, threadlike capsule surrounding the bacteria (C). Unlike the study by Swaney and Breese (1980), the capsule is evident in thin-sections stained only with uranyl acetate and lead citrate. Swaney and Breese had to use a polysaccharide-specific stain to demonstrate the capsule in the streptomycin-resistant CEMO. Perhaps the capsule is denser in the streptomycin-sensitive CEMO, allowing it to bind enough stain to be discernable with the less specific uranyl acetate-lead citrate stain.

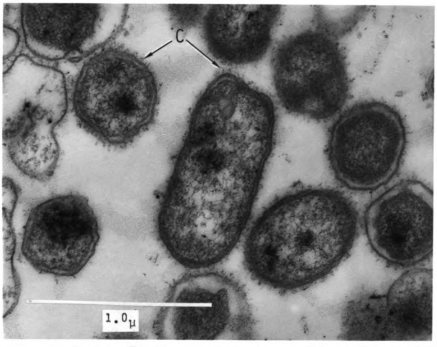

Figure 5. TEM photomicrograph at streptomycin-sensitive CEMO at 49,000X demonstrating a threadlike capsule (C).

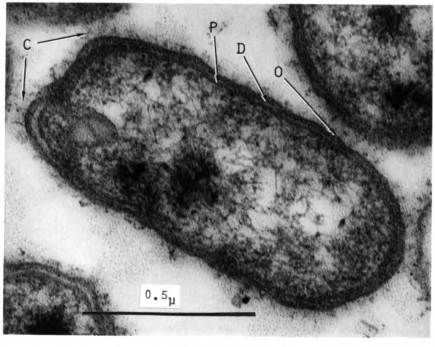

Figure 6. TEM photomicrograph of streptomycin-sensitive CEMO at 91,000X. Labelled features are capsule (C), outer membrane (O), dense intermediate layer (C) and cytoplasmic membrane (P).

The streptomycin-sensitive CEMO is quite similar to the streptomysin-resistant strains in most other morphological characteristics. Both the outer (O) and cytoplasmic (P) membranes have the typical trilaminar appearance. These membranes are separated by a dense intermediate layer (D). There are no signs of pili or flagella in the TEM photomicrographs.

CHAPTER IV

SUMMARY AND SUGGESTED FURTHER STUDIES

4.1. SUMMARY

The animal epidemiological studies found three species with serological test results suggestive of CEMO exposure. Sheep and pig results are suspicious because of the bimodal distribution of PHA titers. Cattle are suspect due to the correlation of serological test results in that group. Both dog and horse groups showed no signs of CEMO exposure.

The human epidemiological studies strongly supported the findings of Taylor and Rosenthal (1978a), suggesting that CEMO or related organisms may be involved in human venereal disease. In both studies, antibodies to CEMO were found three times more frequently in patients being treated for venereal disease than in the normal population.

Correlation of serological test results was found only in cattle. The lack of correlation of test results was a surprise. However, the lack of correlation between some test results can be linked to problems characteristic of test methods used in this study.

The PHA was found to be the most valuable and generally applicable test procedure evaluated. Its ease of performance, theoretical simplicity, universal procedure for all species, and high sensitivity make it an ideal epidemiological screening test.

The scanning EM studies determined that the CEMO has a growth phase-dependent morphology. The transmission EM studies demonstrated the presence of a capsule in the streptomycin-sensitive CEMO strain.

4.2. SUGGESTED FURTHER STUDIES

Further animal studies should include attempts to isolate the CEMO from all mucosal surfaces of serologically reactive animals, concentrating primarily on sheep, pigs and cattle. Bacterial adsorption studies should be performed on reactive sera to identify the specificity of the antibodies detected by serological methods. Similar studies would also be appropriate in serologically reactive humans. In addition, it would be of interest to attempt to correlate human reactivity to specific venereal diseases.

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APPENDICES

APPENDIX A

ANTIGEN PREPARATION FOR SEROLOGICAL TESTS

A.1. CEMO COMPLEMENT FIXATION REAGENT PREPARATION

A.1.A. Antigen Preparation

Plates of chocolatized Eugon agar, made with 10% horse blood, are streaked with a pure culture of CEMO in a manner to yield confluent growth. Plates are incubated at 37°C in 5-10% CO_2 until a lawn of confluent growth is visible, approximately 3-5 days. The CEMO colonies are scraped off the plates and suspended in 250 ml of 0.2% (v/v) formolized C-FIX diluent (tris buffer, pH 7.4). Glass beads are added to the suspension to help break up the clumps of bacteria and the suspension is refrigerated for at least 3 days.

A CEMO suspension is made using a vortex mixer and a tissue grinder. Excessive grinding, which could remove or damage the bacterial capsule, is avoided since the strength of the antigen preparation is proportional to the concentration of CEMO with intact bacterial capsules. The ground CEMO suspension is washed until it is a uniform mixture that can be readily resuspended. This usually requires 3 washes, the first in 0.2% formolized C-FIX diluent and all subsequent in plain C-FIX diluent. After the final wash, the CEMO is resuspended in 250 ml in C-FIX diluent containing 1:10,000 methiolate as a preservative. This concentrated C-FIX antigen must be kept refrigerated.

APPENDIX A (cont'd)

The antigen concentration used in the actual test procedure is determined through preliminary titration of a positive sample with a known endpoint. Various dilutions of the concentrated antigen, on the order of 1:2 up to 1:10, are made in the C-FIX diluent. These dilutions are used to test the known positive sample. The dilution that yields the correct serum endpoint is used for all testing.

A.1.B. Complement Fixation Diluent

Component	Amount/ liter diluent
Tris[1]	1.21 gm
NaCl	8.16 gm
0.15 M $MgSO_4$	3.3 ml
0.10 M $CaCl_2$	1.5 ml
Conc HCl	0.7 ml

Final pH sould be 7.4 +/- .04, adjust down with 0.1 N HCl if necessary.

A.2. CEMO PASSIVE HEMAGGLUTINATION ANTIGEN PREPARATION

A.2.A. Antigen Preparation

Chocolatized Columbia agar plates are inoculated with a pure culture of CEMO in a manner to yield confluent growth. After 5 days incubation at 37°C under 5-10% CO_2, the bacterial growth is scraped from the plates and suspended in PBS (pH 7.2). The CEMO is washed twice in PBS (pH 7.2) and resuspended in PBS (pH 7.2) to 250 mg/ml by wet weight. The suspension

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[1]Trishydroxymethylaminomethane, Sigma Chemical Co., St. Louis, MO.

APPENDIX A (cont'd)

is put into a glass container surrounded by an ice-water slurry and the bacteria disrupted for a total time of 4 minutes using a Bronwill Biosonik III[1] sonicator fitted with a 3/8" probe at the 60% power setting. Intact bacteria and cellular debris are removed by centrifugation at 20,000 rpm for 30 minutes at 4°C. The antigen (supernatant) is stored at -20°C.

A.2.B. Formolized Turkey Erythrocytes

Fresh citrated blood from turkeys is washed in saline until the supernatant is clear and the buffy coat removed. The RBCs are resuspended in PBS (pH 7.2) to a 7% cell suspension. An equal volume of 7% w/v formaldehyde solution in PBS (pH 7.2) is prepared and 1/4 of this volume is added to the 7% cell suspension. The mixture is incubated at 37°C with frequent shaking for 1 hour. The remaining formaldehyde solution is added and the suspension is incubated at 37°C for 24 hours, being shaken frequently during the first few hours. The cells are then washed 10 times in saline and resuspended to a 10% stock suspension in 0.1% w/v azide saline and stored at 4°C.

A.2.C. Cell Tanning and Sensitization

The tannic acid is stored as a 1% w/v stock solution of tannic acid in distilled water and is diluted in saline to a 0.005% w/v solution immediately prior to use. The required volume of 4% tanned cells is prepared by diluting the well-mixed 10% formolized cells with PBS (pH 7.2) to a 4% suspension. To this is added an equal volume of fresh 0.005% tannic

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[1]Bronwill Scientific, Div. of Will Scientific Inc., Rochester, NY.

APPENDIX A (cont'd)

acid solution. This mixture is incubated at 37°C for 15 minutes, shaken once during incubation, centrifuged, washed twice in saline and resuspended in PBS (pH 6.4) to a 4% suspension.

The tanned cells are sensitized by mixing 1 volume of 4% tanned cell suspension with 4 volumes of PBS (pH 6.4) and 1 volume of CEMO antigen diluted to optimal concentration in PBS (pH 6.4). This mixture is incubated for 30 minutes at 37°C, mixing twice during incubation. The suspension is centrifuged, washed once in saline and resuspended in 4 volumes of 1.0% w/v BSA saline to yield a 1% sensitized cell suspension. The control cell suspension, used to detect anti-carrier agglutinins, is prepared as above except PBS (pH 6.4) is used in place of the CEMO antigen.

APPENDIX B

SCREENING DILUTION DETERMINATION FOR PHA

Table B.1. Preliminary PHA titer results

Serum Titer	<8	8	16	32	64	128			
Coded Result	0	1	2	3	4	5	Coded	Coded	Screening
Group							Average	SD	Dilution
Sheep	19	-	-	-	-	-	0.0	-	1:8
Horse	13	5	2	-	-	-	0.45	.69	1:16
Dog	17	2	-	-	-	-	0.10	.42	1:8
Cattle	1	10	6	1	2	-	1.65	1.04	1:32
Pig	20	-	-	-	-	-	0.0	-	1:8
Human	12	-	-	-	-	-	0.0	-	1:8

APPENDIX C

DETERMINATION OF EXACT PROBABILITIES IN A 2x2 CONTINGENCY TABLE

Any 2x2 contingency test based on use of the χ^2 statistic becomes unreliable when the total number tested is less than 40 with any expected frequencies less than 5, or when an expected frequency is very small regardless of the total number tested (Tate and Clelland 1957). In such cases, reliable results can be obtained by determining the exact probabilities, under the null hypothesis, of the observed results and any more extreme cases. The total of these probabilities, converted to percentage, is then subtracted from 100% to give the confidence level at which the null hypothesis may be rejected.

The exact probabilities of the observed and more extreme cases may be calculated using the following procedure. Let A, B, C, and D represent the observed frequencies in a 2x2 contingency table. The marginal totals are fixed under the null hypothesis of independence at A+B, C+D, A+C, and B+D. The probability of the observed values occurring under the null hypothesis is then given by

$$P_O = \frac{(A+B)!(C+D)!(A+C)!(B+D)!}{N!A!B!C!D!}$$

If the next more extreme case has cell frequencies of (A+1), (B-1), (C-1), and (D+1), the probability of this first more extreme case is given by

APPENDIX C (cont'd)

$$P_1 = \frac{(A+B)!(C+D)!(A+C)!(B+D)!}{(A+1)!(B-1)!(C-1)!(D+1)!}$$

but is more effectively computed by

$$P_1 = P_0 \times \frac{BxC}{(A+1)(D+1)}$$

In general, the probability of the next more extreme cases is given by

$$P_{n+1} = P_n \times \frac{(B-n)(C-n)}{(A+n+1)(D+n+1)}$$

Of course, if the next more extreme case has cell frequencies of A-1, B+1, C+1, and D-1, the general probabilities are computed by

$$P_{n+1} = P_n \times \frac{(A-n)(D-n)}{(B+n+1)(C+n+1)}$$

VITAE

The author was born in Milford Michigan on September 3, 1954. She attended Michigan State University where in 1976 she received a B.S. degree in Medical Technology. She completed her professional training at Hutzel Hospital in Detroit Michigan in July 1977. Subsequently she was employed as a medical technologist at the Michigan State University Veterinary Clinical Center. In 1979 she was admitted to the Clinical Laboratory Science program at Michigan State University and received her M.S. degree in Pathology in December 1981. The author is presented employed as a senior medical technologist at the Michigan State University Veterinary Clinical Center. She is married with no children.