IMMUNO FLUORESCENT AND ECOLOGICAL STUDIES OF CORYNEBACTERIUM RENALE

by

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INTRODUCTION

Pyelonephritis and cystitis caused by Corynebacterium renale in cattle primarily and occasionally in other species have been recognized for many years. The organism and the disease it causes have been recognized in this country since the early part of this century, but the overall incidence and economic importance of the disease have not been made evident. This may be due to the fact that many of the affected animals are carrying the infection non-clinically or subclinically while only very few animals really show clinical signs. The organism is very susceptible to all the antibiotics which are used for treatment of other diseases and which can be excreted through the urinary tract. The chronic nature of the disease also makes it inapparent most times and diagnosis can be mistaken for other conditions such as traumatic reticulitis.

Pyelonephritis is observed primarily in dairy cows and seems to affect animals that are over two years and have calved at least once. Other predisposing factors which have been mentioned are injuries to the urogenital tract caused by difficult labor or by catheterization and other genito-urinary tract examinations. High protein diet is considered important due to renal dysfunction.

There seems to be a general ecological limitation as the disease is found more commonly in the temperate countries and the incidence is thought to increase during the winter months of the year. The disease is also prevalent in areas of the world where dairy cows are prevalent.

The clinical signs in typical cases are usually obvious and include straining on urination, frequent urination, hematuria, pain obviated by kicking at belly, treading, arched back and raised tail. Temperature is usually normal. Most infections however go unnoticed because of the nature of the disease.

Little information is still available concerning many aspects of the disease, including route of transmission and pathogenesis. Diagnosis of the disease at the moment is based on the clinical findings on the field, isolation and identification of the organism by biochemical reactions in the laboratory.

The purpose of this investigation was to evaluate the direct immunofluorescent method as a rapid means of identifying C. renale from clinical specimens such as the urine samples or urogenital exudate. One of the more important applications of the FAT is identification of pathogens from clinical specimens without use of prior culture procedure. The routine cultural and biochemical test for microbial infections are long and time consuming. Other serological methods such as precipitation, agglutination, immune adherence hemagglutination, passive hemagglutination, immune hemolyses and immuno diffusion have been used to study C. renale in recent years, but immunofluorescent technique has not been used. This investigation was also designed to study the incidence of shedders, clinical or non-clinical among the cattle visiting the veterinary clinic and to determine the importance of age and calving record to the shedders. Results derived from the study would be used to enhance the understanding of some basic questions about the organism and the disease it produces and for further study on

the biology of C. renale and perhaps of other corynebacteria and how they may be related immunologically.

IMMUNO FLUORESCENT AND ECOLOGICAL STUDIES OF *CORYNEBACTERIUM RENALE*

PAPER 1: IMMUNO FLUORESCENT STUDY OF *C. RENALE*

SUMMARY

The use of the direct fluorescent antibody technique (FAT) for the identification of Corynebacterium renale was investigated. Twenty-two fresh isolates and two stock cultures of C. renale isolated from bovine urinary tract were tested with the conjugate. Excellent specificity was demonstrated since there was no significant cross reaction with related and unrelated organisms.

The technique promises to be of considerable value in the diagnosis of C. renale shedders.

INTRODUCTION

The fluorescent antibody technique (FAT) as introduced and modified in 1941, 1942 and 1950[6, 7, 8] has been applied to a great advantage in many infectious diseases of human and animal origins. One of the more important applications of the FAT is identification of pathogens from clinical specimens without the use of prior culture procedure. The routine cultural and biochemical tests for microbial infection are long and time consuming. This test is rapid and highly specific and is becoming exceedingly important as a diagnostic tool.

Other serological techniques such as precipitation[18], agglutination[12, 15], hemagglutination and immunodiffusion[12] tests, but not immunofluorescent technique, have been used to study Corynebacterium renale.

The present study was designed to develop an immunofluorescent technique for C. renale identification and to use this technique to investigate its specificity.

MATERIAL AND METHODS

Source of Organism

Corynebacterium renale No. 19412 was obtained from American Type Culture Laboratory.* The stock culture was kept as a stab on brain heart infusion (BHI) agar and in BHI broth and was transfered once a week. The stock culture was used as the immunizing antigen. The pathogenicity had been checked by inoculation of sheep.[13]

Preparation of Antigen

Bacteria for immunization purposes were grown on brain heart infusion (BHI) slants for 18-24 hours at 37° C. and harvested in sterile phosphate buffered saline (PBS), pH 7.2. The bacteria were washed three times in sterile PBS. The number of bacteria were estimated both by the optical density and plate counts methods and diluted to approximately 2.35×10^8 viable bacteria per milliliter with PBS.

Preparation of Antiserum

Three white female rabbits weighing approximately 3 kg each were used. The procedure for immunization was similar to that of Yanagawa et al.[18] and each rabbit was given 2.35×10^8 viable cells weekly for 6 weeks following 2 initial doses of dead cells. A total of 8 intravenous inoculations consisting of 2 dead and 6 live were given to each rabbit as recommended by Yanagawa et al.[18]

After the last inoculation, the rabbits were rested for 1 week then bled by cardiac puncture. Serum was collected from each rabbit and stored at -20° C. until ready to use.

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Fractionation of Antiserums

Serum for preparation of FA reagents was fractionated with 50% saturated ammonium sulfate, $(NH_4)_2\ SO_4$, by the method of Kawamura[14]. The amount of protein from each rabbit was determined by the refractometer[a] method. The globulins from the rabbits were then pooled and stored at -20° C until ready to use.

Conjugation

The globulin and fluorescence isothiocyanate (FITC)[b] were conjugated according to the method of Kawamura[14] using 10 μg of FITC/mg of protein. Gel filtration[c] was used to separate the free dye from the conjugate. The dye and protein ratio of the conjugate was determined according to the methods of Goldman.[9]

Preliminary Testing

Preliminary testing was accomplished by using the Corynebacterium renale stock cultures to assess the optimum staining titer of the conjugate. Eighteen hour aerobic growths of the organism on BHI agar were suspended in PBS solution and spread on slides[d] which had been coated with 1% gelatin and air dried. The smeared slides were then fixed for 10 minutes in acetone and dried at 37° C for 5 minutes. The conjugate was serially diluted with phosphate buffered saline (PBS) (pH 7.5).

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[a] American Optical Company Instrument Division, Buffalo 15, New York.

[b] Baltimore Biological Laboratories, Division of Bioquest, Cockeysville, Md.

[c] Sephadex G-25 Fine, Pharmacia Fine Chemicals Inc. Piscataway, N. J.

[d] Trident Fluoro-slide ALDE Scientific, St. Louis 3, Mo.

Four drops of each dilution of the conjugate were spread over a smear and incubated in a moist chamber at 37° C for 45 minutes. The slides were then washed thoroughly but gently with PBS for 15 minutes with 3 changes of the washing solution. They were counterstained 1 minute with diluted (1:20) FA Rhodamine Counterstain.[e] The slides were then washed in PBS for 10 minutes with 2 changes and blotted dry. Buffered glycerin[b] was used as a mounting medium.

The stained preparations were then observed with a F.A. microscope[f]. The average optimum titer with maximum fluorescence at the highest high dry power was observed and recorded for further study.

Adsorption of Conjugate

Corynebacterium pseudotuberculosis isolated from a sheep mediastinal lymph node was used for adsorption. The organism was grown in a flask containing 100 ml of BHI broth on a reciprocal shaker overnight. The cells were harvested in sterile PBS and then washed 3 times and centrifuged[g] for 10 minutes at 5000 r.p.m. (3020 g) each time. The conjugate was tested as previously outlined to determine if C. pseudotuberculosis would cross-react.

Ten milliliters of the conjugate was added to 1 ml of packed C. pseudotuberculosis cells and incubated with frequent stirring for 2 hours in a water bath at 50° C. The conjugate was then centrifuged at

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[e] Difco Laboratories, Detroit, Mich.

[b] Baltimore Biological Laboratories, Division of Bioquest, Cockeysville, Md.

[f] Ernst Leitz, Wetzlar Inc., New York, N. Y.

[g] Sorvall superspeed RC2-B Ivan Sorvall Inco., Newtown, Conn.

5000 r.p.m. (3020 g) for 10 minutes in a refrigerated centrifuge[g]. The supernatant was collected and stored at -20° C until ready to use.

Normal Controls

The normal control consisted of exposing the antigens to pooled normal rabbit globulin collected before the antiserum rabbits were inoculated and which had been conjugated and titrated along with the immune conjugate to demonstrate absence of specific fluorescence occuring naturally.

Tests

Fresh isolates and stock cultures of Corynebacterium renale were tested. Freshly isolated strains were made available from a survey conducted on cows admitted to Dykstra Veterinary Hospital. Stock cultures included strain No. 19412 used for making the antiserum and 3 serotypes from Japan.[18] For specificity studies, some Gram positive and Gram negative bacteria and yeast cells were also tested. A light suspension of each type of bacteria was prepared in PBS, and smears were prepared and examined as previously described.

Autofluorescence

A light suspension of each of the tested organisms was made as previously described. But instead of being stained with the conjugate, they were treated with PBS and conjugate made from normal rabbit globulin. These preparations were examined in a manner similar to the stained preparations.

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[g] Sorvall superspeed RC2-B Ivan Sorvall Inco., Newtown, Conn.

RESULTS

Agglutination titers confirmed the presence of homologous antibody against Corynebacterium renale in the rabbits used for hyperimmune serum production. No rabbit had less than a 1:1024 titer.

Table 1 gives fluorescent antibody results obtained by staining various isolates of C. renale with adsorbed and unadsorbed conjugates and the number of replicates.

The possibility of unsuspected cross reactions with supposedly related and unrelated organisms was investigated by a survey of a broad spectrum of Gram-positive and Gram-negative organisms with the fluorescent antibody conjugate.

The data in Table 2 indicates that cross-reactions with the microorganisms used in this report did not present any serious differential problems.

DISCUSSION

In this study all fresh isolates of Corynebacterium renale that were tested stained with the conjugate and their reactions were similar to stock culture No. 19412 which was used to prepare the immune serum. Only 1 of the 3 serotypes of C. renale from Japan stained strongly with the conjugate. No explanation is offered to why the other 2 did not stain. No other comparison could be made between the isolates of C. renale used in this study and the 3 types from Japan because no attempt was made to serotype the isolates by the precipitin reactions in gel technique[18]. The biochemical characteristics of the cultures from Japan

were not investigated and are not compared with the 22 recent isolates obtained here and the stock culture No. 19412 from American Type Culture Laboratory.

C. pseudotuberculosis is an organism which closely resembles C. renale phenotypically. Since the prime value of FAT is the differentiation of organisms which are similar culturally and morphologically[5] it is significant that only a weak reaction was observed with C. pseudotuberculosis using unadsorbed conjugate. Adsorption of C. renale conjugate with C. pseudotuberculosis therefore appears critical in the utilization of the conjugate.

The moderate staining of the rapid urea positive, nitrate positive but "CAMP"* test negative diphtheroid requires further interpretation. Although these organisms did not show the biochemical characteristics typical of C. renale they do resemble C. renale in their colonial characteristics and in rapidly hydrolyzing urea. They were isolated nearly as often as C. renale from the cows tested, and they may be a closely related organism.

The yellow fluorescence observed in Staphylococcus aureus and S. epidermidis is readily distinguishable from the apple green fluorescence of fluorescein isothiocyanate[9] (FITC).

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* Christie, Atkins and Munch-Petersen.

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Table 1. Fluorescent Reaction of Corynebacterium renale.

Source	Reaction* Unadsorbed	Adsorbed	Replicates
Strain No. 19412 (American Type Culture Lab.)	4+	4+	42
Japan 19**	4+	4+	12
" 20**	1+	0	12
" 21**	1+	0	12
Kansas State University (22 isolates)	4+	4+	5 (each isolate)

* Immunofluorescent Standard: 4+ = maximal specific fluorescence;
3+ = strong specific fluorescence;
2+ = moderate specific fluorescence;
1+ = weak specific fluorescence;
± = slight specific fluorescence;
0 = absence of fluorescence;
A = auto-fluorescence.

** These numbers were arbitrarily used for identification purpose. They do not correspond to the Serotypes I, II and III used by the original workers[12, 18].

Table 2. Organisms Stained with Anti-*Corynebacterium renale* Fluorescent Antibody Conjugate and the Results Using Adsorbed and Unadsorbed Conjugates.

Organisms*	Reaction Unadsorbed	Adsorbed
Corynebacterium equi (6)	0	0
Corynebacterium pyogenes (11)	1+	0
Corynebacterium bovis (2)	0	0
Corynebacterium pseudotuberculosis (ovis) (2)	1+	0
Micrococcus sp. (2)	0	0
Staphylococcus aureus (5)	YF**	YF**
Staphylococcus epidermidis (4)	YF**	YF**
Listeria monocytogenes serotype 5 (1)	0	0
Erysipelothrix insidiosa (rhusiopathiae) (1)	0	0
Bacillus subtilis (2)	†	0
Bacillus cereus (3)	†	0
Streptococcus pyogenes (3)	0	0
Streptococcus fecalis	0	0
Rapid urea +ve, Nitrate +ve CAMP Test negative Diphtheroid (13)	2+	1+
Lactobacillus sp. (2)	0	0
Escherichia coli (4)	†	0
Enterobacter sp. (2)	0	0
Pseudomonas aeruginosa (4)	0	0
Pseudomonas pyocyanea (3)	0	0
Proteus morgani (3)	0	0
Proteus vulgaris (2)	0	0

Table 2 (Continued)

Organisms*	Reaction Unadsorbed	Adsorbed
Neisseria catarrhalis (2)	0	0
Clostridium perfringes (1)	0	0
Clostridium hemolyticum (1)	0	0
Clostridium septicum (1)	0	0
Clostridium chauvoei (1)	0	0
Clostridium tetani (1)	0	0
Pasteurella hemolytica (1)	0	0
Pasteurella multocida (1)	0	0
Salmonella typhimurium (1)	0	0
Unidentified Diphtheroid (6)	0	0
Yeast cells (Candida) (2)	0	0

* Number in parenthesis represented the number of isolates tested.

** YF = Yellow fluorescence.

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PAPER 2: EXPERIMENTAL TRANSMISSION OF *C. RENALE* IN CALVES

SUMMARY

The experimental transmission of Corynebacterium renale infection to calves was accomplished and the microorganisms recovered. These studies included attempts to produce cystitis and/or pyelonephritis, production of shedder calves, hematological changes during infection and persistence of the organism in the urinary tract. The organisms were administered by means of a urinary bladder catheter. C. renale successfully established itself in the urinary tract of the calves but the animals were refractory to clinical infection.

INTRODUCTION

Bovine pyelonephritis is a specific infection of the urinary tract. It is thought to be the most common kidney disease of cattle. The clinical signs vary with organism involved and extent of the infection. There may be passage of turbid or blood stained urine in some cases and in others, the first sign may be an attack of acute colic. Typically the onset is gradual with fluctuating temperature, loss of condition and a decline in milk production. The infection has also been found to exist in nonclinical form[4, 10, 19], and apparently normal animals may shed the organisms in their urine[18, 21]. The disease is thought to be confined to adult animals and usually occurs following a urogenital tract complication such as dystochia, abortion or injury due to catheterization. The organism, Corynebacterium renale, causing this disease was frequently isolated from the vagina and possibly it was a normal inhabitant of the posterior urinary tract of dairy cows.[21]

Many aspects of the disease remain obscured. They include the pathogenicity of pure cultures of the organism, pathogenesis and route of infection.

Earlier attempts to reproduce the disease by experimental infection with pure cultures of the organism were unsuccessful. However, a transient violent inflammation ensued only after the bladder was previously irritated with sterile sand[5, 22]. The pathogenicity of the organism for mice[17] and for rabbits[6, 7] have been studied. In an experimental transmission into mice, a selective action of the organism for the urinary tract was observed, although the organism was given intravenously.[17] It was therefore suggested that the natural disease was another example of tissue localization and infection could be a hematogenous spread. Other investigators, on the other hand, were convinced that the infection was not hematogenous. In these studies the disease was successfully reproduced in adult cows by inoculating the organism directly into the urinary bladder using a sterile urinary catheter[10, 11, 15]. In ewes that were inoculated intravenously the organism was recovered from lung abscesses and synovial fluid but not from urine or urogenital tissue samples taken at post-mortem examinations. None of the ewes had clinical sign nor lesions of cystitis and/or pyelonephritis.[13]

The present investigation was undertaken to determine if calves could be experimentally infected by inoculation through a urinary bladder catheter and if the exposed calves would develop typical signs of pyelonephritis and/or cystitis or if the organism could establish itself in a non-clinical form and the calves becoming carriers.

MATERIALS AND METHODS

Corynebacterium renale Strain

The stock culture of C. renale (strain No. 19412) used in this study was obtained from the American Type Culture Laboratory*. The original organism was isolated from a cow with pyelonephritis**.

The C. renale culture was transfered to a tube of brain heart infusion (BHI) broth and another tube of BHI agar, incubated for 24 hours at 37° C and the tubes were stored in the refrigerator at 5° C for use as stock culture. Subcultures were made weekly following the above procedures. The strain was identified as C. renale by colony characteristics, cell morphology, Gram's stain reaction, biochemical criteria and "CAMP"*** test as described in texts[2, 3, 8]. Pathogenicity was confirmed by inoculation of organisms into sheep[13].

Experimental Animals

Two Holstein heifers approximately 6 months of age and weighing 350 pounds each were purchased locally. The calves were kept in an isolation stall and observed for 2 weeks. Blood samples were drawn 4 times during that period to check their hemograms. Stool samples were checked for gastrointestinal parasites and the number of eggs per gram of feces was determined.

Urine samples were taken from each calf 6 times during the 2 week observation period and the samples were also examined bacteriologically to demonstrate that they were free from previous infection with Corynebacterium renale.

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** J. Gen. Microb., 28, (1962): 35.

*** Christie, Atkins and Munch-Petersen.

Serum samples obtained from the calves prior to inoculation were used for agglutination tests[11] to show that the calves did not produce antibodies capable of neutralizing the organism.

Experimental Transmission

Cultures of Corynebacterium renale were grown on nutrient agar slants at 37° C for 24 hours. The cells were harvested in sterile phosphate buffered saline (PBS) and placed in sterile 50 ml. screw-capped centrifuge tubes*, and centrifuged at 5000 r.p.m. (4080 g) for 5 minutes**, and the supernatant fluid was discarded. The cells were washed 3 times in sterile PBS and then resuspended in sterile PBS and diluted to an optical density of 1.1 as determined by colorimetric method using a Spectronic 20 colorimeter*** at a wave length of 420 mμ. An overnight growth of bacteria often consists of a mixture of living and dead organisms. Thus, estimates of live bacteria will provide different answers from the determination of total bacteria mass. If for example, growth is followed by measuring the turbidity of culture, the figure obtained will include the contribution of the dead organisms. In order to determine the viable cell, a plate count was performed. 0.1 ml. of the suspension of 1.1 was diluted serially to 10^{-12} by 10 fold dilutions using a fresh sterile 1 ml. pipette for each step.

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* Kimble Glass Co., Toledo, Ohio.

** Model CL, International Equipment Co., Needham Heights, Massachusetts.

*** Bausch and Lomb Optical Co., Rochester, New York.

One ml. of each dilution was put on petri dish and about 15 ml. melted nutrient agar cooled to about 40-45° C was added. The petri dish was rotated on the table for a thorough mixing and then allowed to solidify. Each dilution was inoculated in triplicate and the petri dishes remained at room temperature for 30 minutes. They were then incubated at 37° C for 3 days and counted. Only plates having 30 to 300 colonies were counted. The others were discarded as being too numerous to count (TNTC) or too few to count (TFTC). By this method, it was estimated that 1 ml. of suspension at O.D. of 1.1 contained approximately 2.7×10^9 viable cells.

Before the calves were inoculated, the urine was removed from the urinary bladders with sterile catheters. Two ml. of suspension of C. renale containing 5.4×10^9 viable cells was resuspended in 20 ml. of sterile PBS in a 50 ml. syringe and inoculated through the sterile catheter directly into the urinary bladder.

One of the calves had to be inoculated a second time because she stopped shedding C. renale 3 days following the first inoculation. For the second inoculation, the bladder was emptied as above. A caudal epidural block with 2 ml. of 2% Lidocaine* was performed to produce a temporary stasis of urine in the bladder. 200 ml. of 0.2% sterile saline was infused into the bladder through a sterile catheter. The bladder was then emptied 8 hours later, flushed with sterile phosphate buffered saline (PBS) and bacterial suspension was administered as described previously.

The rectal temperatures of the test calves were recorded 2 times a day and the calves were watched for clinical signs of pyelonephritis.

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* McGaw Laboratories, Division of American Supply Co., Glendale, California.

Urine samples were collected daily and examined bacteriologically for C. renale. Blood samples were collected from the jugular veins 2 times a week for complete blood counts.

The calves were examined daily for 4 weeks at the end of which they were treated for 5 days with a daily dose of 5 ml. Procaine Penicillin G.* After treatment urine samples were checked to ascertain the efficacy of the treatment. The animals were held 3 weeks following treatment. They were then sold.

Serum samples taken at the termination of the experiment were observed again by agglutination test for antibodies against C. renale.

RESULTS

Pre-inoculation Studies

Calf 1 had respiratory distress and a conjuctivitis when she was delivered for experimental use. These signs cleared without treatment during the pre-inoculation observations. No gastro-intestinal parasites were found in the samples of feces examined. There were no blood parasites and the serum was negative for antibodies against Corynebacterium renale. Table 1 indicates blood picture during the pre-inoculation phase. This heifer carried unidentified diphtheroid in her urinary tract. It was established by cultural, biochemical and "CAMP" test characteristics that the organism was not C. renale and that the 2 organisms could be properly identified during the experiment.

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* Agricultural Division, Chas. Pfizer and Co., Inc., New York, New York.

Calf 2 had a gastro-intestinal parasite infection (Strongyles) and the egg count per gram of feces was 1,100 (Strongyles eggs). She was treated with Thiabendazole to eliminate the parasites.

Table 2 indicates the pre-inoculation hemogram for calf 2. The urine was negative for C. renale or any diphtheroid and there was no evidence of antibodies against C. renale in the serum.

Both heifers had slight elevated temperatures during the first 3 days following arrival at the experimental site. Their body temperatures returned to normal before inoculation.

Period of Inoculation

In calf 1 Corynebacterium renale established itself in the urinary tract and the animal remained a shedder through 4 weeks of studies. Calf 2 was reinoculated 3 days following the first inoculation. She then remained a shedder throughout the experimental period.

The temperatures of both heifers remained normal throughout the studies and they remained healthy and gained weight. No signs of pyelonephritis and/or cystitis were observed.

The hemograms of calves 1 and 2 remained normal throughout the experimental periods. Serum samples taken from the calves at the termination of the experiment showed no significant level of antibody against C. renale.

DISCUSSION

An attempt was made to establish Corynebacterium renale in the urinary tracts of 2 calves (heifers), each approximately 6 months of age. C. renale was introduced into the urinary bladder of each

heifer. The organism was able to survive in the urinary tracts and both heifers became shedders or carriers. Neither calf developed clinical signs of a urinary tract infection. The establishment of C. renale in these heifers is in agreement with earlier research indicating that infection may occur early in life, but the disease develops in only a proportion of animals.[18]

The results presented here further strengthen the suggestion that the disease is found in adults, following complications such as abortion, dystochia or other injuries to the urogenital tract. In earlier studies cows which were 2 years or older were used[10, 11]. In adult cows it has been observed that it is not easy to reproduce the disease with pure culture[5, 22]. The findings reported here suggest that for purpose of epidemiology, young heifers should be considered as potential carriers and shedders as well as adult cows.

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Table 1. Pre-inoculation Hemogram of Calf 1 Used in a Corynebacterium renale Experiment.

Day	PCV	HB	Total WBC	Absolute Differential Count			
				Neutrophile	Lymphocyte	Monocyte	Eosinophile
1	33	10.3	11000	6930	3410	110	550
5	31	9.4	8200	1968	5822	264	164
9	37	11.8	9500	2945	6460	-	95
14	32	10.7	6900	1242	5106	207	69

Table 2. Pre-inoculation Hemogram of Calf 2 Used in a Corynebacterium renale Experiment.

Day	PCV	HB	Total WBC	Absolute Differential Count				
				Neutrophile	Lymphocyte	Monocyte	Eosinophile	Band
1	28	10	6400	1674	3844	572	62	248
7	31.5	11.3	10000	3800	5400	700	100	-
12	32.0	10.3	10000	3195	6685	120	-	-

IMMUNO FLUORESCENT AND ECOLOGICAL STUDIES OF *CORYNEBACTERIUM RENALE*

PAPER 3: INCIDENCE OF *C. RENALE* IN KANSAS CATTLE

SUMMARY

A total of 29 swabs and 157 urine samples from 186 cows were examined bacteriologically for presence of Corynebacterium renale. The results were compared with clinical signs of urinary tract infection. The results show no significant relationship between clinical signs and shedding of C. renale.

Of the 22 cows (11.83%) shedding C. renale, only 2 (9.09%) showed clinical cystitis or pyelonephritis.

In addition to C. renale, 13 diphtheroid isolates closely related to C. renale were cultured from 13 cows without clinical signs of cystitis and/or pyelonephritis. Other common species of Corynebacterium were also isolated.

INTRODUCTION

The incidence of Corynebacterium renale infection in cattle has been reported a number of times previously in this and other countries[2, 3, 8, 12, 18, 22, 25, 27]. Prior studies along this line have demonstrated that the organism may occur in both healthy and clinically ill animals[8, 12, 13, 21, 24, 27]. The route of infection with the organism is controversial[8, 21, 22, 23].

It was suggested that the natural disease was just another example of tissue localization and that there was nothing inconsistent or unusual in postulating a hematogenous spread[23]. Other investigators were convinced that the infection was not hematogenous since cows inoculated intravenously with cultures did not develop pyelonephritis

while smaller quantities of cultures introduced into the bladder through the urethra produced typical persistent infection with clinical signs [20, 21, 22]. Some authors successfully reproduced the disease in adult cows by innoculating the organism directly into the urinary tract using a sterile catheter[13, 14, 15, 16].

Although the disease entity is recognized in this country, the precise economic significance has not been established. Recent reports of the findings in Japan[13, 14, 15, 16] suggest the disease could be significant here also.

It has been suggested that infection of the urinary tract due to C. renale is more common with bovine female than in the male[11, 13, 14, 15, 16].

There has not been a similar study on the incidence of C. renale infection among Kansas cattle. The purpose of the study reported here was to determine the incidence of C. renale in the cattle referred to the Dykstra Veterinary Clinic, College of Veterinary Medicine, Kansas State University, for urogenital problems or other disease conditions and to correlate the findings with clinical signs of urinary tract infection.

MATERIAL AND METHODS

As urinary tract infections and pyelonephritis caused by Corynebacterium renale are believed much commoner in the female than in the male, this study was limited to female cattle. One hundred and eighty-six cows aged 6 months to 12 years were studied. One hundred and eighty-six specimens consisting of 157 urine specimens and 29

vaginal swabs were obtained for Gram's stain and culture. The urine samples were collected by the naturally voided technique and vaginal swabs were taken when it was not possible to obtain urine specimens from the cows. Each cow was sampled once and within 24 hours of arrival at the Dykstra Veterinary Hospital.

Each sample was labelled and the clinical history of each cow recorded. Urine samples were collected in sterile 100 ml collecting bottles and closed with screw-caps and the swabs with Culturette*. Tests were started as soon after collection of samples as possible. Samples which were not examined and cultured immediately were refrigerated until they were examined. No samples were stored for more than 24 hours before tests were commenced. A 20-50 ml sample of urine was centrifuged** in screw capped centrifuge tubes at 5000 r.p.m. (4080 g) for 5 minutes. The supernatant was discarded and sediment redissolved in the remaining milliliters of supernatant. One loopful of the dissolved sediment was plated on blood agar (BA), phenylethanol agar (PEA) and MacConkey agar. The vaginal swabs were similarly plated on BA, PEA and MacConkey plates and the plates were incubated aerobically for 24 hours at 37° C. The remaining sediment and swabs were smeared on microscope slides and stained with Gram's stain. The morphologically positive or negative reactions of the organisms were recorded for each case.

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* Medi/Flex Division, Medical Supply Company, Rockford, Illinois 61101.

** Model Cl, International Clinical Centrifuge. International Equipment Co., Needham HTS, Massachusetts.

After 24 hours of incubation, the plates were examined and representative colonies were selected for subculture and identification. Identification of selected colonies was based on Gram's stain reactions, cell morphology, colony appearance and biochemical reactions according to Bergey's Manual and other standard texts[4, 6,] and methods[10].

RESULTS

Over a 6 month period, samples were collected from 186 cows representing 8 cattle breeds. Vaginal swabs and urine specimens were used in the study. In 33 samples, Gram's stain reactions were negative. The total number of C. renale positive specimens are illustrated in Table 1.

Table 2 illustrates the number of cows shedding C. renale and presence or absence of clinical signs of disease.

Data from Table 2 indicates two cows or 9% of the total number of shedders had clinical signs of cystitis and/or pyelonephritis. The remainder either had other urogenital problems or in most cases no clinically detectable urogenital problem.

Data from Table 3 indicates that most of the shedders were cows which had calved at least once, and their ages varied from 2 years to 11 years. Five heifers were 1 year or less than 1 year old.

Table 4 illustrates the numbers of cattle representing each breed and the numbers and percentages positive.

Table 5 represents the data on other pathogens isolated in association with C. renale. In addition to those organisms regarded as pathogens, other non-pathogenic organisms such as C. bovis, Staphylococcus epidermidis, α hemolytic streptococci, non-hemolytic Escherichia

coli and Enterobacter aerogenes were isolated in association with C. renale from the bovine urogenital tract.

Table 6 indicates the genera of organisms isolated from the urine and vaginal swabs and the frequency of the isolates. In most cases, the classification was taken to the species level.

Considering the methods of collection of specimens and contamination of the posterior genital tract with feces, the relatively high number of recoveries of enteric bacteria particularly E. coli and alpha hemolytic Streptococcus is not surprising. The presence of beta hemolytic E. coli in these samples may be important since they are capable of causing infection of the urogenital tract.

Most of the organisms listed in Table 6 are potential pathogens. Most noticeable is the relatively high incidence of C. pyogenes. This organism was isolated from 11 out of 186 cows.

DISCUSSION

Corynebacterium renale was isolated from 22 of the 186 cases examined. Although this shows a high percentage of infection (11.83%), it is believed that the incidence may be higher. Many of the cows examined were cases referred to the Dykstra Veterinary Hospital by veterinarians in the field. Some were cows which had received antibiotics or other bactericidal drugs before they were admitted. It is possible that some of the animals were cleared of C. renale before they were admitted. In Japan an infection rate of 13.3% from urine samples and 8.0% for vaginal smears was reported[14]. The results of the present study compare favorably with those of previous workers (11.40% for

urine and 13.79% for swabs). Less than 10% of the carriers showed clinical signs. The infection rates of C. renale in apparently healthy cows, reported by other workers were also high[14, 27, 28]. The percentage from apparently normal cows in this study is 10%. This comparatively lower figure could be due to the fact that some of the cows could have received antibiotics prior to admission.

Some of the cows (about 7%) carried a pigmented, rapid urea positive, nitrate positive but "CAMP"* test negative diphtheroid. These organisms morphologically and colonially resemble C. renale. They did not show typical reactions of C. pseudotuberculosis[3, 5, 10, 17, 24]. Recent work suggests that C. renale hydrolyzed urea faster than any other animal corynebacteria and most other urease-positive bacteria.[1] If this is true, these isolates are either true C. renale which are nitrate positive and "CAMP" test negative or they are corynebacteria which closely resemble C. renale.

It has been suggested that infection with C. renale may occur early in life, but the disease develops in only a portion of the animals[23]. Five of the 22 animals reported here which carried C. renale were female yearlings which had never been bred and without calving records indicating an early life infection.

It was not possible to compare, other than generally, these findings with those of earlier workers because samples were not taken from specific herds and no attempt was made to type the isolates into types I, II, and III[14, 27].

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* Christie, Atkins and Munch-Petersen Test.

Because of the wide range of organisms isolated, there is no evidence in this study that C. renale alone is the cause of clinical pyelonephritis. Most of the animals examined in this study were referred to the clinic for non-urogenital problems.

Because of the relatively high incidence of C. renale and other equally potential pathogens in urogenital tract of cows clinicians should consider urogenital tract conditions in many cases not necessarily with typical syndromes.

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Table 1. The Incidence of *Corynebacterium renale* Positive Samples by Vaginal Swab or Free Voided Urine.

Type of Specimen	Total No. Examined	Total No. Positive	Percent Positive
Urine	157	18	11.40
Vaginal swab	29	4	13.79

Table 2. C. renale Positive Cattle and Associated Urogenital Disorders or Other Signs.

Animal No.	Associated Urogenital Disorder	Disorder other than Urogenital
4	None	Traumatic reticulitis
5	"	Traumatic reticulitis
12	"	Brought in for vaccination against Brucellosis
39	"	Invasive carcinoma
40	Pyelonephritis	None
49	Dystochia	Caesarian Section
53	Dystochia	Caesarian Section
61	Pyelonephritis	None
70	Rupture of the uterus	Toxemia
77	None	Vaccination against Black Leg
96	"	Displaced abomasum
97	"	None
98	"	Overgrown hoofs
112	"	Emaciation, off feed
118	"	Listeriosis
121	"	Displaced abomasum
139	"	Intestinal obstruction
146	"	Vaccination against Brucella
150	"	"

Table 2 (Continued)

Animal No.	Associated Urogenital Disorder	Disorder other than Urogenital
157	None	Overgrown hoofs
170	Prolapse of the uterus	None
172	Vaginal prolapse	None

Table 3. History of Calving, Age and Breed of 22 Cows Positive for *Corynebacterium renale*.

Cows No.	Age	Breed	Gestations
4	4 Years	Holstein	Two
5	6 "	"	Several
12	Less than 1 Year	Hereford	None
39	10 Years	"	Several
40	7 "	Holstein	Several
49	2 "	Angus	1st Calving
53	2 "	Hereford	1st Calving
61	5 "	Holstein	Several
70	5 "	Hereford	Several
77	Less than 1 Year	"	None
96	8 Years	Holstein	Several
97	5 "	"	"
98	5 "	"	"
112	6 "	"	"
118	2 "	"	1st Calving
121	6 "	"	Several
139	4 "	"	Two
146	Less than 1 Year	Hereford	None
150	" " 1 "	"	"
157	" " 1 "	"	"
170	2 Years	"	1st Calving
172	11 "	"	Several

Table 4. Breed Distribution of 186 Cows Sampled for *Corynebacterium renale*.

Breed	No. Examined	No. Positive	Percent Positive
Holstein	58	11	19.00
Hereford	91	10	10.99
Angus	20	1	5.00
Jersey	6	0	0
Charolais	4	0	0
Brown Swiss	3	0	0
Guernsey	2	0	0
Mixed	6	0	0
TOTAL	186	22	

Table 5. Pathogenic Bacteria Found in Association with *Corynebacterium renale* Isolated from Vaginal Swabs and Urine Specimens from 22 Cattle.

Cow No.	Bacteria isolated in association with *C. renale*
4	Unidentified diphtheroid*
5	**
12	*Corynebacterium equi*
39	**
40	Hemolytic *Escherichia coli* and unidentified Diphtheroid* *Staphylococcus aureus*
49	*S. aureus*
53	*S. aureus*
61	*C. pyogenes*, *S. aureus*, Non-hemolytic *Streptococcus*
70	Beta hemolytic *Streptococcus*
77	**
96	*S. aureus*
97	**
98	*S. aureus*
112	**
118	Non-hemolytic *Streptococcus*
121	*S. aureus*
139	Non-hemolytic *Streptococcus*
146	**
150	**
157	Beta-hemolytic *Streptococcus*

Table 5 (Continued)

Cow No.	Bacteria isolated in association with C. renale
170	**
172	Unidentified diphtheroid*

* Pathogenicity has not been checked.

** C. bovis; Non-hemolytic E. coli, Enterobacter aerogenes, alpha hemolytic Streptococcus and Staphylococcus epidermidis which were considered non-significant.

Table 6. The Frequency of Isolation of Particular Microorganisms from the Urinary Tracts of 186 Female Cattle.

Organism Isolated	The Genera and Frequency of Isolation		
	Urine	Vaginal swabs	
	No. of times isolated	No. of times isolated	Total No. of isolates
Bacillus sp (G+ rods)	23	6	29
Staphlococcus aureus	12	3	15
S. epidermidis	24	7	32
Beta hemolytic Streptococcus	6	1	7
Alpha hemolytic Streptococcus	98	22	120
Non-hemolytic Streptococcus	11	2	13
Micrococcus sp	8	3	11
Corynebacterium renale	18	4	22
C. pyogenes	10	1	11
C. equi	8	1	9
C. bovis	17	4	21
Nitrate positive, Rapid urease positive CAMP test negative diphtheroid	10	3	13
Other unidentified diphtheroid	17	3	20
Lactobacillus sp	9	2	11
Sarcina sp	2	1	3
Candida sp	3	-	3
Hemolytic Escherichia coli	21	2	23
Non-hemolytic E. coli	81	27	108
Enterobacter sp	17	4	21
Aeromonas sp	3	-	3

Table 6 (Continued)

Organism Isolated	The Genera and Frequency of Isolation		
	Urine No. of times isolated	Vaginal swabs No. of times isolated	Total No. of isolates
Alkaligenes fecalis	1	-	1
Pseudomonas aeruginosa	3	-	3
P. pyocyanea	4	-	4
Proteus vulgaris	2	1	3
Proteus morgani	4	1	5
Neisseria catarrhalis	14	1	15
Unidentified G-rods	2	-	2

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APPENDIX

REVIEW OF LITERATURE

HISTORY

Corynebacterium renale was first observed as a pathogen in 1877 by Damman in a typical case of pyelonephritis. Hess in 1890 also observed curved bacilli in urine and kidneys of cows affected with pyelonephritis. He described the signs and lesions of the disease and classified it to clinical types. Bollinger in 1890 named the organism Bacillus renalis bovis. Enderlein (1891) cultured it in artificial medium. In the same year Höflich also isolated the organism and named it Bacillus pyelonephritides bovum. Ernst (1905) described and changed the name to Corynebacterium renalis. The organism was later classified as Corynebacterium renale (Breed et al. 1957).

The organism and the disease it causes was known for many years in Europe before it was first described in the United States of America by Boyd (1918). Later, extensive work was done between 1925 and 1930 by Jones and Little of the Rockefeller Institute.

Biological Studies of Corynebacterium renale and the Disease it Produces

Pyelonephritis is a specific infection of the urinary tract and typically it is a chronic infection but occasionally runs an acute course (Blood and Henderson 1968). It is thought to be the most common kidney disease of cattle. Pyelonephritis is primarily a disease of cattle but has been reported in dogs by Olafson (1930), in horses by Boyd and Bishop (1937), in buffaloes by Mohanty and Singh (1970), and in other species by Merchant (1935).

The clinical signs of pyelonephritis in cattle as described by Jubb and Kennedy (1970) and Blood and Henderson (1968) vary considerably with clinical type. There may be passage of turbid or blood stained urine and in other cases the first sign may be an attack of acute colic. More often, the onset is evidenced by slightly elevated and fluctuating temperatures, loss of condition and a decline in milk production. Jones and Little (1925-1930), Weitz (1947), Morse (1950), Hiramune et al. (1970) and several other investigators have reported that the disease also exists in a nonclinical form and that apparently healthy animals can carry the organism in their urinary tract. These animals are reported to act as carriers and are the source of potential infection for susceptible cattle.

The method of infection and the route taken by the organism in the individual case have been the subject of much study. The route of infection is very controversial. Ernst (1905) could not reproduce the disease by experimental infection with pure cultures of the organism when the cultures were inoculated by means of a urinary bladder catheter. He questioned the ecological relationship of the diphtheroid to the disease processes encountered and the mode of infection by the organism. In another effort to reproduce the disease experimentally, he first injected a sterile sand into the bladder of cows to irritate the bladder and on the following day 100 ml of broth culture. Violent inflammation ensued but subsided after nine days. The experiments with negative results led him and others to question the relationship of diphtheroid to pyelonephritis. The work on experimental infection in mice by Lovell and Cotchin (1946) indicated a selective action of the

organism for urinary tract although the organism was given intravenously. It was therefore suggested by Lovell (1959) that the natural disease was another example of tissue localization and that there was nothing inconsistent or unusual in postulating a hematogenous spread. Jones and Little (1925, 1926, 1930) did extensive work on the biology of the organism and the disease it produces. From their findings, they were convinced that the infection was not hematogenous since cows inoculated intravenously with pure cultures of C. renale did not develop pyelonephritis while small quantities of the cultures introduced into the bladder through the urethra produced typical persistent infection with clinical observations. Similar studies were conducted by Hiramune et al. in 1971 and 1972. These investigators successfully reproduced the disease in adult cows by inoculating live cultures of C. renale directly into the urinary bladder using a sterile urinary catheter. Ivogli (1972) experimentally inoculated ewes intravenously and recovered the organism from lung abscesses and synovial fluid but not from urine samples taken during antemortem examinations or urogenital tract tissues examined at postmortem.

The first comprehensive study of the epidemiology and ecology of C. renale infection in this country was conducted by Jones and Little (1930). They examined and cultured various segments of the urinary tract of several bulls and heifer calves. Jones and Little reported that the organism successively established itself in the vagina, penile sheath and urethra without involving the bladders and the kidneys. Weitz (1947) working independently in Britain reported a 40% incidence in dairy cows. Morse (1950) surveyed a number of apparently normal

adult dairy cows around Ithaca, New York, during an 18-month period to determine rate of infection with C. renale, and reported a 17.6% infection with a median carrier incidence of 22.7% in herds with pyelonephritis and 6.7% in herds with no history of pyelonephritis.

Hiramune et al. (1970, 1971, 1972) studied the epidemiological pattern in Japan. Boyd (1918), Boyd and Bishop (1937) and Zdelar (1968) prepared case reports on C. renale infections over a period of several years in their localities.

Merchant (1935) studied 20 isolates of corynebacteria associated with diseases of domestic animals including C. renale. He found that C. renale was variable in its fermentation of carbohydrates, although all the strains fermented glucose. Jones and Little (1926) studied 26 strains of C. renale from disease cattle and they found that 25 strains were similar regarding morphology and cultural characteristics. They were also related serologically using agglutination reactions and limited number of agglutinin absorption tests. Similarly Brooks and Hucker (1944) made a comprehensive study of 79 cultures of diphtheroid organisms from animal sources of which 17 were original isolates and 62 from other laboratories. They observed that it was futile to attempt to devise a key system on any but arbitrary grounds since no high degree of correlation could be found in the characters studied. They grouped the diphtheroids into 3 groups as active, inactive and intermediate. C. renale according to that classification belonged to the intermediate group. They suggested that further splitting of these groups into small units should be based on character other than those used in their study since the characters they used did not show sufficient importance and magnitude to allow such subdivision.

Fraser (1964) examined the effect of combination of diffusible substances produced by bacteria on animal erythrocytes. He observed that diffusible substances from Staphylococcus aureus and C. renale increased the hemolytic effects of the substances on ruminant erythrocytes and this "CAMP"* test was useful as a diagnostic method for the detection of corynebacteria and identifying the different staphylococcal hemolysins including a previously unknown lysin that was found in certain coagulase-positive strains of canine origin.

A more recent study of the biology of C. renale was conducted by Yanagawa et al. (1967). Based on their study, the organism has been separated into three types, I, II and III by the use of precipitin reactions in gels, using 78 isolates of C. renale for their study. These 3 types have been found not only different in their serological behaviors but also in their abilities to produce clinical infections. Hiramune et al. (1970) observed that there was a relationship between the distribution of the 3 types in the herds with and without histories of occurence of pyelonephritis. These investigators (Yanagawa et al. 1967 and Hiramune et al. 1970) observed that type I strains were isolated from apparently healthy cows, type II strains were from both normal urine and the material of pyelonephritis. Type III strains were isolated always from cows affected with clinical pyelonephritis.

In addition to the above there was variation in sex distribution. Type I existed more frequently in male than female, type II existed in both males and females without significant difference and type III was found nearly always in females. Type I was most predominant

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* Christie, Atkins and Munch-Petersen.

and type III was the least predominant in number of isolates. More recent studies of the 3 types were made by Honda (1972), who observed by deoxyribo-nucleic acid (DNA) homology studies that the three types were related but the relation of type I to type II was closer than that of type II to type III. This information may help to explain some of the failures and confusions experienced by earlier investigators in their attempts to differentiate between pathogenic and non-pathogenic types of C. renale.

Fluorescent Antibody Studies

The fluorescent antibody technique (FAT) as introduced by Coons et al. (1941, 1942) and Coons and Kaplan (1950) has been applied successfully in many infectious diseases of both human and animal origin. The FAT is an immunochemical tool and its function is based on the principles governing immunological reactions involving antigens and antibodies. It allows direct observation and the precise localization of the reaction between small amounts of antigen and antibody. This reaction may be observed on or in cells and in frozen or free-dried tissue section or smears (Coons and Kaplan 1950) in paraffin embedded tissue, (Sainte-Marie 1962) or in formalin fixed tissues, (Cook et al. 1971).

One of the more important applications of the FAT is identification of pathogens from clinical specimens without use of prior culture procedures. The routine cultural and biochemical tests for microbial infection are long and time consuming. The FAT is upheld for its rapidity and specificity. It is not surprising therefore that it is

becoming exceedingly important as a diagnostic tool. Lovell (1946) studied C. renale serologically by the agglutination and agglutinin absorption tests. Other serologic tests have also been used (Yanagawa et al. 1967 and Hiramune et al. 1972). Fluorescent antibody tests have not been used to identify the organism and to study the relationship among several isolates.

MATERIAL AND METHODS

Origin and Maintenance of Corynebacterium renale Strains

The strain No. 19412 of C. renale used for the experimental aspects of this study was originally isolated from a cow with pyelonephritis.* The stock culture, strain No. 19412, was obtained from the American Type Culture Collection** in lyophilized form. Opened cultures were grown on brain heart infusion (BHI) agar slant and in tubes of BHI broths. Culture tubes were preserved in the refrigerator at 5° C. Cultures were transfered once a week following the above procedures. The strain was identified as C. renale by colony characteristics, cell morphology, Gram's stain and the Christie, Atkins and Munch-Petersen (CAMP) test. Pathogenicity was confirmed by inoculation of the organisms into sheep (Ivogli 1972).

Anti-Corynebacterium renale Serum

Anti-C. renale serum was prepared in rabbits according to the procedures employed by Yanagawa et al. (1967). Three female rabbits, weighing approximately 3 kg. each, were used. They were inoculated intravenously at 7 day intervals, with a total of 8 inoculations consisting of the first 2 with dead cells and the last 6 with live cells.

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* J. Gen. Microb. 28, (1962): 35.

** 12301 Parklawn Drive, Rockville, Maryland 20852.

Table 1. Immunization Schedule for Preparing Corynebacterium renale Antiserum in Rabbits

Day	Optic Density	Viable count per ml.	Total number of cells given
1	0.3	None (killed culture)	4.70×10^8 (=2 ml cell suspension)
4	"	" " "	4.70×10^8 "
7	"	2.35×10^8	2.35×10^8 (-1 ml cell suspension)
14	"	" "	2.35×10^8 "
21	"	" "	4.70×10^8 (=2 ml cell suspension)
28	"	" "	" " "
35	"	" "	" " "
42	"	" "	" " "

The rabbits were rested for 7 days following the last inoculation. They were then exsanguinated by cardiac puncture. The serums were collected by conventional methods and were kept separate until they were tested by the agglutination test according to Hiramune et al. (1972) and found to contain homologous antibodies against C. renale.

Cultures for inoculation were prepared as follows. For the formalin killed antiserum C. renale was grown on tube slants of brain heart infusion agar overnight at 37° C. The cells from the slants were collected in a sterile tube containing 10 ml of 0.02% formalin-phosphate buffered-saline (PBS). The absorbance of the cultures was read at 420 mu in a Baush and Lomb Spectronic 20[a] and diluted with formalin PBS

[a] Bausch and Lomb Optical Co., Rochester, New York.

until absorbance was 0.3. The cell suspension in formol PBS was then placed in the incubator at 37° C for 48 hours, followed by overnight in the refrigerator at 5° C. After treatment as outlined in the procedures above, loopfulls of the cell suspension were plated in BHI agar plates to ascertain that the cells were dead and no other bacterial contaminants were growing.

For the live antigen, the weekly inoculations were grown overnight on BHI slants and harvested in sterile PBS and standardized as outlined above to absorbance of 0.3 in a spectrophotometer with sterile PBS.

An overnight growth of bacteria typically consists of a mixture of living and dead organisms. Thus estimate of live bacteria will vary if determination is based on the total bacteria mass. If for example growth is determined by measuring the turbidity of the culture, the growth figure obtained will include the contribution of any dead organisms present. In order to determine the viable cell percentage a plate count was performed.

0.1 ml of the cell suspension in PBS was diluted serially. Ten-fold dilutions were made up to 10^{-12} using a fresh sterile 1 ml pipette for each step. Then 1 ml of each dilution was put in a petri dish and 15 ml of melted nutrient agar cooled to 40-45° C was then added. The petri dish was rotated on the table for thorough mixing and then allowed to solidify. Each dilution was done in triplicate and the petri dishes remained at room temperature for 30 minutes. They were then incubated at 37° C for 3 days and counted. Only plates having between 30 and 300 colonies per plate were counted. It was found that

1 ml of suspension at optic density of 0.3 contained about 2.35 X 10^8 viable cells.

Agglutination Tests for *Corynebacterium renale* Antibody

The antibody titers of the hyperimmune serums produced in rabbits were determined by the agglutination test. The method was similar to that of Hiramune *et al.* (1972) with modification in the method of bulk growth. The antigen was prepared by growing *C. renale* in brain heart infusion broth at 37° C for 36 hours. Incubation was carried out aerobically by means of a reciprocal shaker. The bacterial cells were harvested by centrifugation in the Sorvall super-speed centrifuge[b] at 5000 r.p.m. (3020 g) for 15 minutes. The cell pellets were washed 3 times with distilled water to free them of adhered material. After the third washing the pellets were resuspended in distilled water in a test tube and boiled for 15 minutes. The test tube was left at room temperature overnight and the suspension which remained above the naturally sedimented bacteria was removed and the suspension was centrifuged and washed 3 times at 5000 r.p.m. (3020 g) 30 minutes[b] with phosphate buffered saline (PBS). The total bacterial mass was determined by mg wet weight. The pellets were resuspended in phenol PBS (PBS containing 0.5% Phenol). The final concentration of the bacteria in phenol PBS was adjusted to 2 mg/ml and used as antigen for the agglutination test.

Serial two-fold serum dilutions (0.5 ml) were mixed with equal volume of the antigens in test tubes. The tubes were incubated at room

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[b] SORVALL Superspeed RE 2-B automatic centrifuge. Ivan Sorvall Inc., Newtown, Conn. 06470.

temperature overnight and then read. A second series of tubes were incubated at 37° C for purpose of comparison of the reactions at room temperature and at 37° C. Titers were expressed as the highest dilution showing definite agglutination. No significant difference was observed at the agglutination reactions either at room temperature or at 37° C.

Fractionation of Corynebacterium renale Antiserums

The antiserums were pooled and fractionated according to the methods of Goldman (1968), Nairn (1969) and Kawamura (1969). All materials used in fractionation of globulins were cooled in a refrigerator for 24 hours before use and the procedure was carried out at room temperature with the flask containing the anti-serum in an ice bath.

The pooled anti-serum was diluted two-fold with 0.1 M phosphate buffered saline (PBS) (pH 7.2), poured into a flask and gently stirred with a magnetic stirring bar[c] on a magnestir[d]. An equal volume of saturated ammonium sulfate $(NH_4)_2SO_4$ solution was added drop by drop. The mixture was allowed to stand for 1 hour with continuous stirring in a refrigerator (5° C). It was then centrifuged[b] at 10000 r.p.m. (12,100 g) for 15 minutes, using 50 ml screw-capped pyrex centrifuge tubes[e]. The supernatant was discarded and the sediment redissolved to its original volume in PBS. Precipitation with ammonium sulfate was

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[c & d] Cole-Parmer Instrument Co., 7425 Oak Park Ave., Chicago, Ill. 60648.

[e] Kimble Glass Co., Toledo, Ohio.

repeated 3 times. The final precipitate was redissolved in 1.5 ml of PBS and dialized in a cellophane tube in a refrigerator against glass distilled water for one hour and then against PBS for 2 days with replacement of the PBS 4 times a day and constant stirring of the dializing solution until all the NH_4^+ and $SO_4^=$ ions were removed. The solution was checked for the presence of $SO_4^=$ by adding 50 mg of barium chloride ($BaCl_2$) to 1 ml of the dializing solution. A white precipitate was formed because of the PO_4 ion from the PBS but the turbidity disappeared on acidification with 0.1 M hydrochloric acid (HCl) indicating the precipitate was not due to $SO_4^=$ ions. Following dialysis, the test solution was checked for its protein concentration with a protein refractometer[f]. The protein was then stored in test tubes at -20° C until used.

Preparation of Fluorescein-labelled Globulin

The protein solution obtained from salting out procedure described above was adjusted to 2% with 0.5 M sodium carbonate-bicarbonate buffer, pH 9.5. 0.01 mg of fluorescein isothiocyanate[g] (FITC) was added to each mg of protein by dissolving 1/100 FITC to the amount of protein weight in 1 ml 0.5 M sodium carbonate-bicarbonate buffer pH 9.5, adding the dissolved FITC slowly using a Pasteur pipette and adding at 1 drop at a time and stirring with magnetic stirring bar on a magnestir. The procedure was carried out in a refrigerator at 5° C for 4 hours allowing adequate conjugation to occur.

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[f] TS Meter. American Optical Corporation, Buffalo, N.Y. 14215.

[g] Baltimore Biological Laboratories, Division of Bioquest, Cockeysville, Md.

Removal of Free Dye

Unconjugated fluorescein dye was removed from the conjugated globulin solution by passing it through a Sephadex column G-25 fine[h] equilibrated with 0.005 M phosphate buffered saline (PBS) pH 7.2. The Sephadex column was prepared as follows. Twenty gms of fine Sephadex G-25 was added to 200 ml PBS and the fine particles were removed. The process was repeated three times and the Sephadex particles were then allowed to swell in the last 200 ml of PBS for 3 hours at room temperature and were then poured into the column. The conjugated globulin was applied to the column and eluted with the PBS buffer used to equilibrate the column.

Adsorption of the Conjugate for Improved Specificity

Bulk growth of Corynebacterium pseudotuberculosis (ovis) was grown and harvested as described earlier for Corynebacterium renale. One ml of packed cells was then mixed with 10 ml of conjugate and incubated with frequent stirring for 2 hours at 50° C. The conjugate was then centrifuged[b] at 5000 r.p.m. (3020 g) for 10 minutes. The supernatant was collected and used as the adsorbed conjugate.

Titration of the Adsorbed and Unadsorbed Conjugates

To determine the optimum dilution of the conjugates a light suspension of Corynebacterium renale No. 19412 in phosphate buffered saline (PBS) was smeared on slides[i]. Serial dilutions of the conjugate were

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[h] Pharmacia Fine Chemicals Inc., 800 Centennial Ave., Piscataway, N. J. 08854.

[i] Trident fluoroslides for FA Examination. ALDE Scientific St. Louis 3, Missouri.

made at 1:2, 1:4, 1:8, 1:16, 1:32, 1:64, 1:128 and used to stain the smears. Optimum dilution was determined on the basis of a maximum (4+) fluorescence with minimum background fluorescence at 400 magnifications (10 X eye pieces, 160 mm tube and 40 X objective).

Testing the Conjugate for Specificity Utilizing Several Genera of Bacteria

Twenty-two fresh isolates of Corynebacterium renale isolated from bovine urine and vaginal swabs from Dykstra Veterinary Hospital, C. renale types I, II and III from Japan and stock culture No. 19412 used for making the hyperimmune serum were tested with the conjugates. In addition to the strains of C. renale, certain Gram positive and Gram negative bacteria and some yeast cells generally associated with diseases of cattle and other species were tested.

Staining Procedure:

Chemically clean slides were first coated with 1% gelatin solution and allowed to air dry. A light suspension of each genus or species of bacteria was prepared in 0.1 M phosphate buffered saline (PBS) and smears were made with a wire loop within the etched circles on the slides. The smears were allowed to air dry. The circles were marked with a Mark Tex-Tech. Pen[j]. The air dried smears were fixed in acetone for 10 minutes and dried in an incubator at 37° C for 5 minutes.

Drops of the previously diluted conjugate were placed in each inscribed circle and spread to cover the entire smear with applicator sticks. The slides were incubated in moist chambers (plastic petri dishes containing moistened filter papers) at 37° C for 45 minutes. The stained slides were washed thoroughly but gently with PBS for 15

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[j] Mark-Tex Corporation, Englewood, N. J.

minutes with 3 changes of the washing solution. They were counterstained for 1 minute with diluted (1:20) FA Rhodamine Counterstain[k], washed 10 minutes with PBS with 2 changes of the PBS. The slides were blotted dry. Buffered glycerin[g] (pH 7.5) was used as a mounting medium. Stained preparations were examined immediately with an FA microscope[1] utilizing an Osram HBO 200w light source, BG 38 heat absorbance and KP 430 interference filters and Leitz K510 barrier filter.

Autofluorescence and Normal Controls Procedures:

The normal control method consisted of staining the antigens with pooled normal rabbit globulin taken and prepared prior to exposure of the rabbits to Corynebacterium renale antigens and was conjugated and titrated along with the immune conjugates to demonstrate lack of specific fluorescence naturally to the antigens. In addition the antigens were treated with phosphate buffered saline (PBS) instead of normal or immune conjugates to check for specific autofluorescence. All treated slides were examined the same way.

Experimental Transmission of Corynebacterium renale Infection in Calves

Two Holstein heifers approximately 6 months of age and weighing 350 lb. each were obtained locally. Urine samples contained no C. renale and serum samples were free of C. renale antibodies by the agglutination test. The heifers were inoculated by means of sterile urinary catheters with a total of 20 ml of suspension containing 5.4×10^9

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[k] Difco Laboratories, Detroit, Michigan.

[1] Ernst Leitz, Wetzlar Inc., 468 Park Avenue, South New York, New York.

viable cells directly into the urinary bladder. Morning and evening rectal temperatures were recorded daily. Urine samples were collected daily for bacteriological examinations. Blood samples were taken two times a week from each heifer for hemogram studies. All clinical signs were closely monitored. Serums taken at the termination of each experiment were examined for C. renale antibodies.

Survey of Female Cattle for the Detection of Shedders

One hundred and eighty-six female cattle aged 6 months to 12 years were studied. One hundred and eighty-six specimens consisting of 157 urine specimens and 29 vaginal swabs were obtained for Gram's stain and bacterial cultures. The cows were those admitted to the Dykstra Veterinary Hospital College of Veterinary Medicine, Kansas State University, for varying medical problems and urine samples were collected by the natural voiding technique. When it was not possible to collect urine samples vaginal swabs were made.

Samples were plated on blood agar, MacConkey agar and phenolethanol agar and incubated at 37° C. The plates were examined daily for 3 days and colonies were studied morphologically and biochemically.

Fluorescent Microscopy

A Leitz Ortholux research microscope[1] equipped for transmitted light fluorescence with 4 mm BG 38 heat absorbing filter, KP 430 interference filter, K510, K530 barrier filters, and an immersion darkfield

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[1] Ernst Leitz, Wetzlar Inc., 468 Park Avenue South New York, New York.

condensor D1.20 was used. An Osram mercury vapor lamp[m] HBO 200W L-2 was used as the source of light. For examinations of the preparations 4X, 10X, 25X and 40X objectives and 10X eye pieces were used. For photomicrography a 54X oil immersion objective and 12.5X eye pieces were used. A Leitz orthomat 35 mm automatic microscope camera and Kodak Tri-X Pan high speed black and white film ASA 400/DIN27 was used.

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[m] E. Leitz Inc., Rockleigh, New Jersey 07647.

RESULTS

Table 2. Titration Studies on Anti-*Corynebacterium renale* Conjugates for Purposes of Obtaining Optimum Fluorescence with Minimum Non-specific Background Staining.

Titer	Reaction
1: 2	4+
1: 4	4+
1: 8	4+
1:16	4+
1:32	2+
1:64	1+
1:128	0

Table 3. Cultural and Biochemical Characteristics of Diphtheroid Organisms Isolated from Urine of Calf No. 1 Prior to Inoculation with Corynebacterium renale.

Tests	Reaction
Growth on Blood agar	+
" " MacConkey	0
" " Phenylethanol agar (PEA)	+
" at 25° C	+
" " 37° C	+
" " 42° C	+
Colonial morphology	Pale white, moist, raised, smooth
Cell morphology	Palisading rods
Gram Stain	Gram +ve
Spore test	0
Catalase	4+
Oxidase	0
O_2 requirement	+
Motility	0
H_2S Butt	0
Indole	0
Nitrate	+
Urea	0
Simmon citrate	0
Gelatin	0
Litmus Milk	0

Table 3 (Continued)

Tests	Reaction
Cetrimide	0
Oxidative/Fermentative (OF)	0
10% Dextrose	Acid
10% Lactose	Alkaline
Fermentation (after 3 days)	
Mannitol	0
Maltose	0
Sucrose	0
Lactose	0
Xylose	0
Dextrose	0
"CAMP" Test*	Slight Enhancement
M. R.	0
V. P.	0

* Christie, Atkins, Munch-Petersen Test.

Table 4. Hemogram of Calf No. 1 During Experimental Period Following Inoculation with *Corynebacterium renale*.

Day (PI)*	Hb	PCV	Total WBC	Absolute Differential Count			
				Segmenters	Lymphocytes	Monocytes	Eosinophiles
0**	10.8	35.0	6533	2025	4312	65	131
2	11.0	33.0	7786	1012	5995	234	545
5	11.4	32.0	10327	2065	7229	723	310
10	10.2	31.0	10244	2766	6863	410	205
15	11.0	33.0	10295	2265	6588	721	721
20	11.2	33.0	10231	2865	6752	614	-
24	10.6	30.0	10000	1200	8600	200	-
28***	11.0	30.0	10705	3212	7172	321	-

* PI Post inoculation

** Blood sample collected immediately after inoculation

*** " " " last day of experiment just before treatment with Penicillin began.

Table 5. Daily Rectal Temperature Calf No. 1 During Baseline Studies (Preinoculation Studies).

Day	A.M.	P.M.
1	-	102.8
2	103.4	103.0
3	102.8	102.8
4	102.6	102.4
5	101.6	101.2
6	101.6	101.8
7	102.0	101.9
8	101.8	101.8
9	101.8	102.0
10	101.8	101.6
11	102.0	102.0
12	101.8	101.6
13	102.0	102.0
14	101.8	102.0
15	102.2	102.0

Table 6. Daily Rectal Temperature of Calf No. 1 During the Experimental Period Following Inoculation with Corynebacterium renale.

Day	A.M.	P.M.
1	101.4	101.6
2	102.0	101.8
3	102.0	102.0
4	101.8	102.0
5	102.0	101.8
6	101.8	101.6
7	101.6	101.6
8	101.4	101.8
9	102.2	101.6
10	101.8	101.6
11	102.0	101.8
12	102.0	101.8
13	101.6	102.2
14	101.8	102.0
15	101.4	101.8
16	101.4	101.4
17	102.4	101.8
18	102.2	101.8
19	101.6	101.6
20	102.2	102.0
21	101.8	101.8

Table 6 (Continued)

Day	A.M.	P.M.
22	101.6	101.6
23	101.8	102.0
24	102.0	101.8
25	101.8	102.0
26	101.8	102.2
27	102.2	102.2
28	102.0	101.8

Table 7. Hemogram of Calf No. 2 During Experimental Period Following Inoculation with *Corynebacterium renale*.

Day (PI)*	Hb	PVC	Total WBC	Absolute Differential Count			
				Segmenters	Lymphocytes	Monocytes	Eosinophiles
0**	10.8	33.0	9717	2624	6608	97	388
2	12.0	35.0	9855	1971	7490	296	98
5	10.9	32.0	9788	4209	5285	196	98
10	11.0	32.0	8700	3045	5220	348	87
15	10.2	30.0	9595	2591	6812	192	-
20	10.2	31.0	9343	1401	6914	1028	-
24	10.4	32.0	9156	1465	7416	183	92
28***	10.2	30.0	8770	1930	6665	175	-

* PI Post inoculation.

** Blood sample collected immediately after inoculation.

*** " " " last day of experiment just before treatment with Penicillin began.

Table 8. Daily Rectal Temperature of Calf No. 2 During Base Line Studies (Pre-inoculation Studies).

Day	A.M.	P.M.
1	-	102.0
2	102.6	103.0
3	103.4	103.0
4	104.2	104.4
5	103.0	102.8
6	102.4	102.0
7	101.8	101.8
8	101.8	102.0
9	102.2	102.0
10	102.0	101.8
11	101.8	101.4
12	101.4	101.2
13	101.4	101.4
14	101.0	101.0
15	101.0	101.0

Table 9. Daily Rectal Temperature of Calf No. 2 During Period Following Experimental Inoculation with Corynebacterium renale.

Day	A.M.	P.M.
1	101.0	101.0
2	101.0	101.0
3	101.0	101.0
4	101.0	100.9
5	101.0	101.0
6	101.0	101.2
7	101.2	101.2
8	101.0	101.0
9	101.0	101.0
10	101.4	101.2
11	101.4	101.2
12	101.6	101.4
13	101.4	101.4
14	101.0	101.2
15	101.2	101.0
16	101.4	101.0
17	101.4	101.2
18	101.0	101.2
19	101.0	101.0
20	101.0	101.0
21	101.0	101.0

Table 9 (Continued)

Day	A.M.	P.M.
22	101.0	101.0
23	101.2	101.0
24	101.0	101.0
25	101.0	101.9
26	101.2	101.0
27	101.0	101.0
28	101.9	101.0

Table 10. History and Microbiological Data Form Used for the *Corynebacterium renale* Study in Cows.

Date_____ Specimen No._____ Species_____

Breed_____ Age_____ Sex_____ Use_____

History including recent treatment; Herd size Clinic No._____
Location of Herd_____
(County)

Bacteriological Exams: Media, colony type, gram stain action and Hemolysis.

1.
2.
3.
4.

Biochemical Reactions

Reactions	Isolates				Reactions	Isolates			
	1	2	3	4		1	2	3	4
Catalase					KCN				
Oxidase					Gelatin				
Mortility					Glucose				
TSI					Lactose				
Nitrate					Mannitol				
Indole					Maltose				
M-R					Sucrose				
V-P					Litmus Milk				
Urease					CAMP				
Citrate					OTHERS				

Table 11. "CAMP" Test for Identification of Staphylococcal Beta-lysin and the Differentiation of Diphtheroid Organisms.

	C. equi	*C. pseudotuberculosis*	*C. renale*	*C. pyogenes*	*L. monocytogenes*	*E. insidiosa*	*S. aureus*
Corynebacterium equi		E		E	E		E
Corynebacterium pseudotuberculosis	E		E	E	E		I
Corynebacterium renale		E					E
Corynebacterium pyogenes	E	E					E
Listeria monocytogenes	E						E
Erysipelothrix insidiosa							
Staphylococcus aureus	E	I	E	E	E		

E = Enhancement of the hemolytic action of Beta-lysin.

I = Inhibition of the hemolytic action of Beta-lysin.

Table 12. Cultural and Biochemical Characteristics of Corynebacterium renale-related Diphtheroids Isolated from Bovine Urinary Tracts.

Tests	Reaction
O_2 requirement	+
Growth on Blood agar	+
" " MacConkey	0
" " Phenylethanol agar	+
Pigment (colony)	Yellow
Growth at 25° C	+
" " 35° C	+
" " 42° C	+
Colonial characteristic	Dry, flat
Cell morphology	Palisading rods
Gram stain action	Gram +ve
Spore test	0
Catalase	+
Oxidase	0
Motility	0
H_2S Butt	0
Indole	0
Nitrate	0
M. R.	0
V. P.	0
Urea	+ (rapid)

Table 12 (Continued)

Tests	Reaction
Simmon citrate	0
Gelatin	0
Litmus milk	0
Oxidative/Fermentative (OF)	F
10% Dextrose	Acid
10% Lactose	Alkaline
Fermentation (after 3 days)	
Mannitol	0
Maltose	0
Sucrose	0
Xylose	0
Detrose	Acid
"CAMP" Test*	0

* Christie, Atkins and Munch-Petersen Test.

EXPLANATION OF PLATE I

Fig. 1. Hemolytic effects obtained on bovine blood agar by drawing cultures of *Corynebacterium renale* strains horizontal to a downward streak of a Beta-toxin producing *Staphylococcus aureus*.
Top left, *C. renale* No. 19412.
Bottom left, *C. renale* Japan 19.
Center right, *C. renale* obtained from cow No. 12.
Note the increased hemolytic effects of the 3 strains of *C. renale* on *S. aureus*.

Fig. 2. Hemolytic effects obtained on bovine blood agar by drawing cultures of *C. renale* strains horizontal to a downward streak of a Beta-toxin producing *S. aureus*.
Top left, *C. renale* Japan 20.
Bottom left, *C. renale* Japan 21.
Center right, *C. renale*, obtained from cow No. 12.
C. renale from cow No. 12 enhanced the hemolytic effect of *S. aureus*. Japan No. 20 and 21 had no effect.

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PLATE I

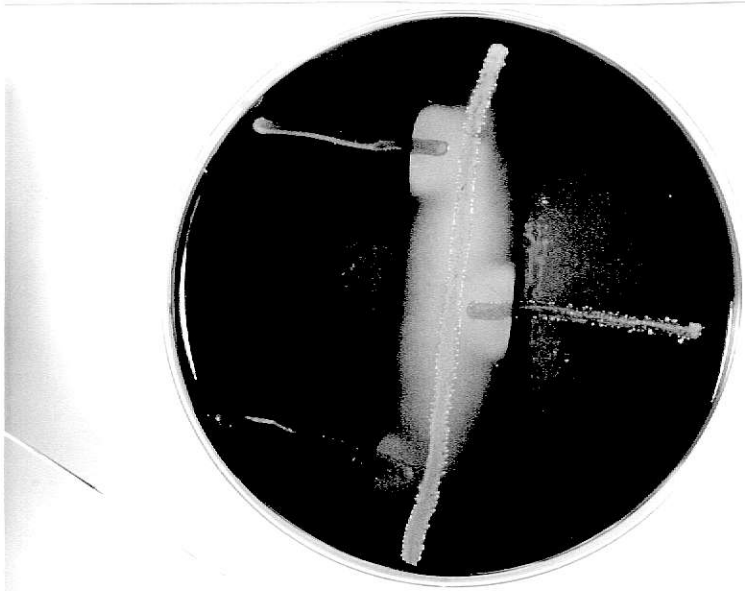

Fig. 1

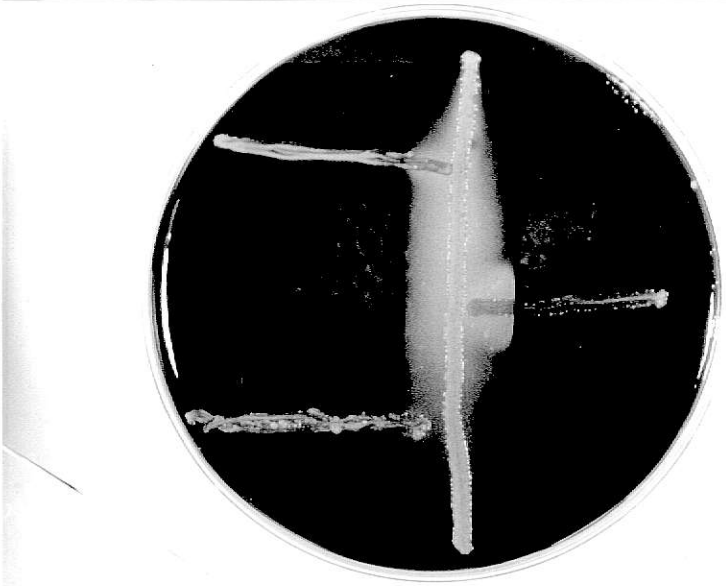

Fig. 2

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EXPLANATION OF PLATE II

Fig. 3. Hemolytic effects obtained on bovine blood agar by drawing cultures of C. pseudotuberculosis, C. renale and C. renale related horizontal to a downward streak of a B-toxin-producing S. aureus.
Top left, C. pseudotuberculosis.
Bottom left, C. renale related diphtheroid.
Center right, C. renale from cow No. 12.
Combination of diffusible substances formed by C. pseudotuberculosis and S. aureus inhibited the hemolytic effect of S. aureus. The effect of the staphylococcal B-toxin was potentiated by C. renale from cow No. 12 but was not affected by C. renale related diphtheroid.

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PLATE II

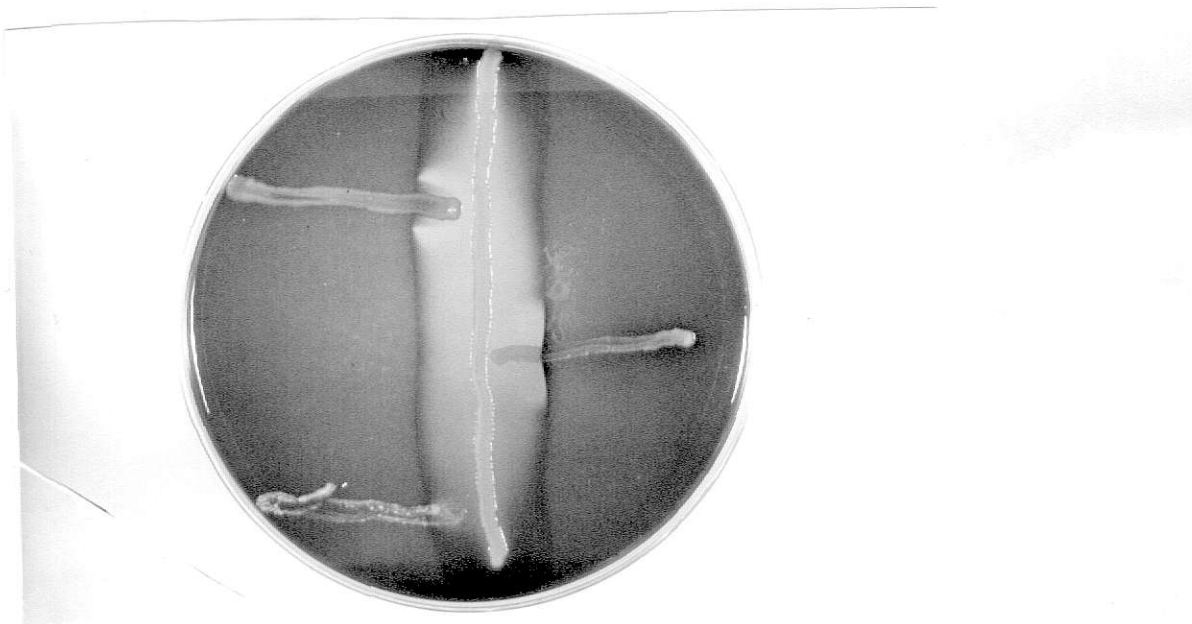

Fig. 3

EXPLANATION OF PLATE III

Fig. 4. *Corynebacterium renale* No. 19412 stained with adsorbed conjugate; X 1620.

Fig. 5. *C. renale* isolated from cow No. 12 stained with adsorbed conjugate; X 1620.

PLATE III

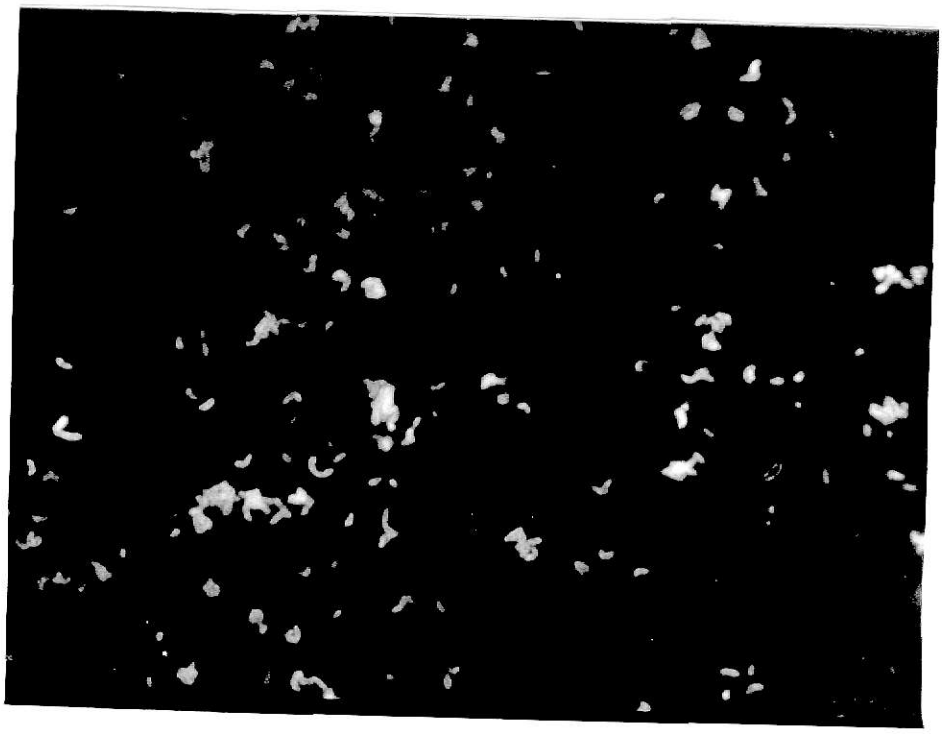

Fig. 4

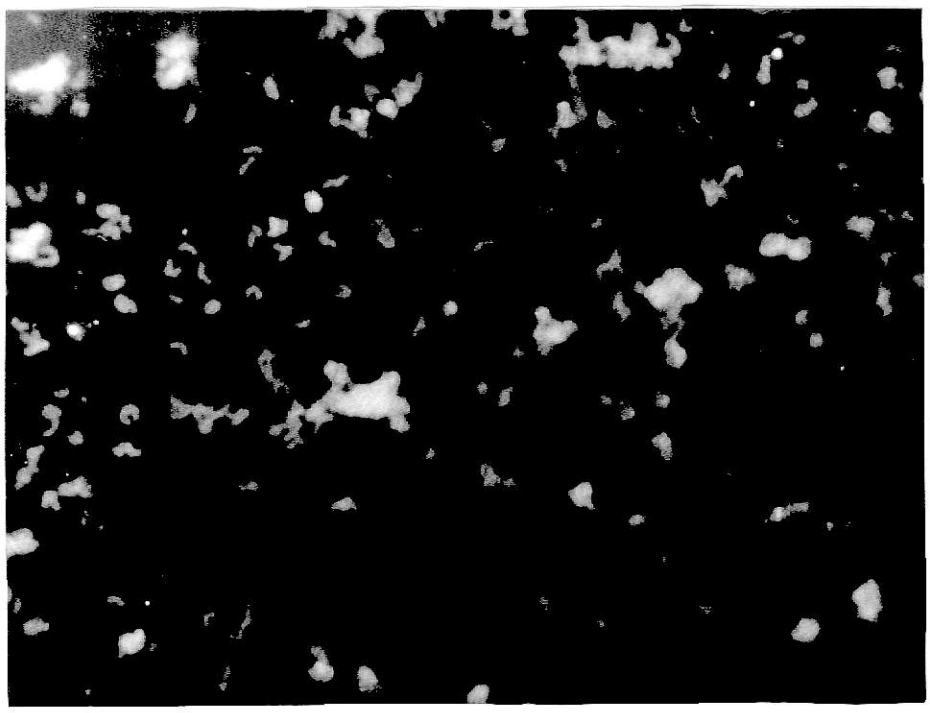

Fig. 5

EXPLANATION OF PLATE IV

Fig. 6. Corynebacterium renale Japan 19 stained with adsorbed conjugate; X 1620.

PLATE IV

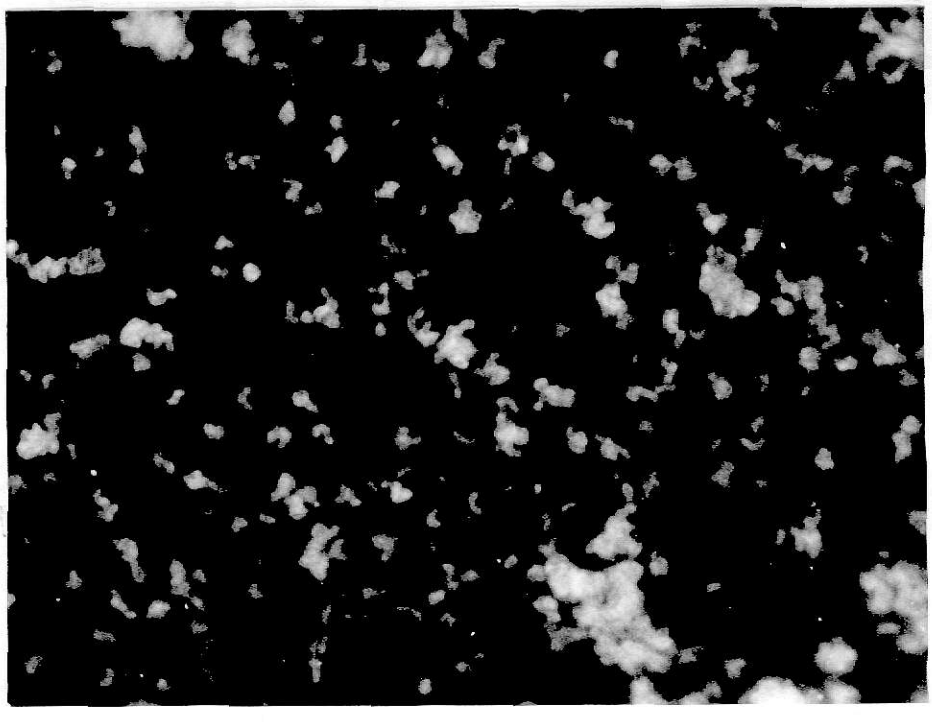

Fig. 6

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IMMUNO FLUORESCENT AND ECOLOGICAL STUDIES OF *CORYNEBACTERIUM RENALE*

by

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AN ABSTRACT OF A MASTER'S THESIS

submitted in partial fulfillment of the

requirements for the degree

MASTER OF SCIENCE

Department of Pathology

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Corynebacterium renale is reportedly a pathogen primarily of cattle and occasionally of other species such as dogs, horses and pigs. It is thought to be non-pathogenic for primates. The ecology of the disease produced by the organism is rarely studied and the use of fluorescent antibody technique for the identification of the organism or the diagnosis of the disease had not been reported.

The present study was designed for the following reasons:

1. To prepare a fluorescein isothiocyanate tagged antibody preparation against C. renale and to use the conjugate to stain the organism.
2. To use the same conjugate to stain other corynebacteria and some related and unrelated bacteria and so determine the degree of specificity of the conjugate.
3. To detect clinical and nonclinical shedders of C. renale among selected Kansas cattle coming to Dykstra Veterinary Hospital.

Urine specimens and vaginal swabs were obtained from 186 cows brought to the hospital between Jan. 15 and June 15, 1973, and were examined for C. renale by culture techniques. Urine specimens were collected from cows as they voided naturally. Samples were centrifuged at 5000 r.p.m. (4080 g) for five minutes. Specimens of sediment from each sample were plated on blood agar containing 5% bovine blood, MacConkey agar and phenolethanol agar plates, incubated at 37° C. and examined for C. renale and other bacteria. Identification was based on colony morphology, Gram's stain, cell morphology and biochemical reactions.

In order to ensure reliability of the bacteriological techniques for recovering C. renale two heifers approximately 6 months of age and each weighing 350 lb. were experimentally infected with C. renale culture No. 19412 obtained from American Type Culture Collection. C. renale No. 19412 was readily recovered from the urine of both heifers over a period of four weeks. The results suggest a non-clinical early infection in calves and the possibilities of carriers animals in the natural transmission of C. renale infection.

The fluorescent antibody technique (FAT) identification of C. renale was investigated. The FAT staining reactions of the organism was compared with the existing agglutination and precipitation reactions and the diagnostic cultural technique. The FAT had a broader spectrum of activity than the corresponding precipitation reaction. The conjugate made from a single strain of C. renale stained all confirmed isolates of the organism at the same dilution and specificity of conjugate was good.