MYCOPLASMA MELEAGRIDIS INFECTION

SLIDE STUDY SET #13

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INTRODUCTION

Mycoplasma meleagridis (MM) was first isolated in 1958 from turkey poults with air-sac lesions. Being antigenically distinct from M. gallisepticum, which was the mycoplasma of primary concern in turkeys at that time, the new serotype was named "N-strain." Since then, the organism has been classified into the H-serotype and given the present species name. The clinical syndrome of airsacculitis and/or associated skeletal abnormalities has been referred to as "day-old-type" airsacculitis and "turkey-syndrome-'65" (TS-65).

DISTRIBUTION AND INCIDENCE

MM has a world-wide distribution, being found wherever turkeys are raised. It is a specific pathogen of turkeys and causes a high incidence (20-65%) of airsacculitis in day-old poults.

PATHOGENICITY AND ASSOCIATED DISEASE CONDITIONS

The disease manifestations and economic losses due to MM are associated with embryo infection via egg transmission and include (a) late incubation (25-28 days) mortality of hatching eggs; (b) bone deformities, particularly during the first 3 to 6 weeks of life; (c) a decrease in livability and growth rate (related to item b above; and (d) condemnation at processing of fryer-roasters due to airsacculitis. Recent studies with experimental and naturally infected eggs suggest that MM causes hatchability losses of approximately 5%. The skeletal problems (TS-65) include bowing,

twisting, and shortening of the tarsometatarsal bones, hock-joint swelling, and deformity of the cervical vertebrae. Stunting and poor feathering may accompany the disease. Affected flocks may show a 5 to 10% incidence of gross lesions. Mortality is due primarily to cannibalism.

Although the organism invades the upper (sinus and trachea) and lower respiratory tract, lesions are confined to the air sacs. Consequently, rales and sinusitis are not observed, and the newly hatched poult with extensive airsacculitis shows no clinical signs. Lesions, which first develop in the thoracic air sacs, progress to the abdominal air sacs by the third to fourth week of age. If uncomplicated, the lesion regresses in 15 to 16 weeks of age. MM interacts synergistically with M. synoviae to produce sinusitis and with M. iowae to produce a more severe airsacculitis.

Despite the high egg-transmission rate of MM in infected flocks (average of 25% over a season's lay), the skeletal deformities do not always accompany embryo infection, and condemnation at processing is not necessarily a consequence of MM airsacculitis. What determines whether an embryo will develop skeletal problems is not clear, but such factors as MM strains of varying pathogenicity and environmental stress during hatching or brooding may influence the disease picture. Secondary bacterial or viral infections may contribute to mixed air-sac infections, resulting in condemnation of fryer-roasters.

HISTOPATHOLOGY

Despite extensive invasion of the organism into many sites, significant microscopic changes are seen only in the air sacs and lungs of embryos 25 to 28 days old. These include heterophil and mononuclear cell infiltration accompanied by various amounts of fibrin and cellular debris. In 2 week-old poults infected experimentally via the air sac, lymphocytic perivascular infiltration and fibrinocellular exudate are seen in 2 days followed by lymphoid follicle formation by 16 days.

The skeletal lesions of TS-65 are similar to the long-bone lesions seen in perosis of dietetic origin. Thus, the main lesion is seen in the proximal end of the long bones, where the cartilage farthest from the blood vessels descending into the proliferative zone from the cartilaginous epiphysis lacks cell density and contains abnormal-appearing chondrocytes.

Gross lesions due to MM invasion are not seen in the reproductive organs of either sex. However, lymphofollicular foci, including plasma cells and heterophils, are seen in the submucosa of the fimbria, uterus and vagina of infected adult hens. In the male, an extensive lymphofollicular infiltration is seen in the region of the mucous-type glands in the submucosa of the lymphfolds of the phallus.

TRANSMISSION

MM is essentially a venereally transmitted disease. Poults of either sex hatched from infected eggs may carry the organism in

their reproductive organs, which persists through sexual maturity. At sexual maturity, when semen from phallus-infected males is pooled and used to inseminate the hens by artificial insemination (AI) the female population becomes rapidly infected (oviduct infection). Also, the organisms can be transmitted on AI straws from infected to uninfected oviducts. Within 2 to 3 weeks, egg transmission begins and continues throughout the lay period.

MM may be transmitted directly or indirectly at any stage of the turkey's life. Direct airborne transmission from infected to uninfected turkeys may occur within a hatchery, within a flock, or on occasion between flocks separated by a quarter of a mile. Airborne transmission usually results in a high infection rate (up to 100%), which remains localized in the sinus and trachea; in young turkeys (brooding and growing period), however, the organism may localize in the genitalia of approximately 5% of the turkeys infected by the respiratory route.

Indirect transmission results from management practices including sexing, palpation of hens, artificial insemination, and vaccination, whereby mycoplasma is manually carried from infected to uninfected turkeys via contaminated hands, clothing, and equipment. Both direct and indirect transmission result in inapparent infections, and unlike egg transmission, such modes of transmission usually do not lead to any of the disease manifestations mentioned earlier.

DIAGNOSIS

When monitoring flocks for MM infection, specimens from the trachea, cloaca, oviduct, or phallus of live turkeys may be taken on sterile cotton swabs for culture. The source of the specimen will vary with the age of the turkeys. If the turkeys are to be necropsied, the specimens should be taken from various parts of the respiratory and reproductive systems and the bursa of Fabricius. Although MM may be isolated easily from many sites of an infected egg, the yolk or lung-air sacs are usually sampled for culture. After the egg is opened aseptically, sterile cotton swabs are used to take specimens.

A number of culture media are available for MM isolation, including Frey's media with swine or horse serum, which are described in the companion slide sets on avian mycoplasmosis. We have had excellent results with media prepared from Difco PPLO base (Table 1). A satisfactory procedure for primary isolation of MM consists of placing the cotton swab with the specimen onto an overlay enrichment medium. After incubation for 4-6 days, the culture is streaked on to agar medium. The latter is placed in a sealed container with added moisture and incubated at 37 C for 3 to 7 days before being examined for mycoplasma colonies under a dissecting microscope. Direct streaking of swabs on agar media could be also done.

The cultures are screened for their cholesterol requirement (digitonin test) and ability to utilize arginine and glucose (Table 1). Cloned colonies should be used to inoculate the differential

media to eliminate the possibility of a mixed culture, because other species of mycoplasmas may be found in the same tissues as MM. MM cultures are digitonin sensitive, arginine positive and glucose negative. While these tests are useful for presumptive identification of MM, the definitive procedure is based on the fluorescent-antibody or growth-inhibition test.

Reliable serologic tests are available for the serodiagnosis of MM infection. These include the rapid plate (RP), rapid plate dilution (RPD), tube and microagglutination, and micro-hemagglutination-inhibition (micro-HI) tests and the micro-enzyme-linked immunosorbent assay (micro-ELISA). When these tests are used in proper combinations, the problems of false-positive and negative reactions are minimized. Usually, the RP test results are confirmed by one or more of the other procedures.

CONTROL AND ERADICATION

Since MM is an egg-borne infection and economic losses are associated primarily with such infections, it has been a common practice to dip commercial turkey eggs in solution of tylosin (up to 3,000 ppm) or gentamicin sulfate (up to 1,000 ppm). The procedure is not completely effective in eliminating the organism, but it does reduce the incidence of infection, leading to improved hatchability and growth performance.

More recently, emphasis has shifted from control of MM in commercial meat turkeys to eradication of the organism from primary breeders. The egg-treatment procedure, which has shown promise,

consists of a dual procedure of dipping of eggs in tylosin (2,500 ppm) and/or gentamicin sulfate (750 to 900 ppm) followed by injection of a solution containing 0.6 mg of gentamicin and 2.4 mg of tylosin per dose into the small end of the egg. Depending on the strain of turkeys, the treatment regime may depress hatchability by 10% or more. It would thus be prudent to determine in a pilot study the effect of such a treatment on hatchability of eggs from a new strain of turkey before large-scale treatment programs are initiated. Serologic and cultural monitoring of turkeys from treated eggs are conducted at about 16 weeks of age and periodically thereafter. Since the treatment regime has high efficacy, monitoring turkeys before 16 weeks would be of little value unless all of the turkeys are tested. The latter would be impractical, because the smallest treatment groups consist of about 10,000 turkeys. The potential for direct and indirect transmission must be considered carefully when planning a program of MM eradication, as these are probably the most difficult aspects of MM transmission cycle to control.

A program of MM testing and certification was adopted at the 1980 National Poultry Improvement Plan Conference, from which a classification, "U.S. M. meleagridis clean", became effective as of January 1, 1983.

In view of the high prevalence of MM in the turkey population, a program of test and slaughter has not been feasible for MM eradication, as was the case for the elimination of M. gallisepticum. Some data indicate that the dual treatment

procedure used to eradicate MM is also effective for eliminating M. synoviae and M. gallisepticum.

SELECTED REFERENCES

1. Adler, H.E., J. Fabricant, R. Yamamoto and J. Berg. Symposium on chronic respiratory diseases of poultry. I. Isolation and identification of pleuropneumonia-like organisms of avian origin. Am. J. Vet. Res. 19:440-447. 1958.
2. Edson, R.K. Mycoplasma meleagridis infection of turkeys: motivation, methods, and predictive tools for eradication. PhD Thesis. Univ. Calif., Davis. 166 p. 1980.
3. Freundt, E.A., H. Erno and R.M. Lemcke. Identification of mycoplasmas. In: Methods in Microbiology, Chapt. IX, T. Bergman, and J.R. Norris, eds. pp. 377-434. Academic Press, N.Y. 1979.
4. Ghazikhanian, G.Y., R. Yamamoto, R.H. McCapes, W.M. Dungan and H.B. Ortmayer. Combination dip and injection of turkey eggs with antibiotics to eliminate Mycoplasma meleagridis infection from a primary breeding stock. Avian Dis. 24:57-70. 1980.
5. Madoff, S. Isolation and identification of PPLO. Ann. N.Y. Acad. Sci. 79:383-392. 1960. (reference to Dienes' stain).
6. National Poultry Improvement Plan (NPIP), 25th Conference, July 8-10, 1980. New Orleans, La. Adopted changes to the plan. BARC-East Bldg. 265, Baltimore, Md 20705. R.D. Schar, Senior Coordinator.
7. Ortmayer, H.B. A cultural field screening procedure for detection of Mycoplasma meleagridis in the reproductive

tract of turkeys. M.S. Thesis. Univ. of Calif., Davis. 100 p. 1970.

8. Ortmayer, H.B., and R. Yamamoto. Mycoplasma meleagridis antibody detection by enzyme-linked immunosorbent assay (ELISA). Proc. 30th Western Poult. Dis. Conf., Mar. 9-11. Davis, Calif. 1981.

9. Rhoades, K.R. Mycoplasma meleagridis infection: development of lesions and distribution of infection in turkey embryos. Avian Dis. 15:762-774. 1971.

10. Rhoades, K.R. Turkey airsacculitis: effect of mixed mycoplasmal infections. Avian Dis. 25: 131-135. 1981.

11. Saif, Y.M., K.E. Nestor and K.E. McCracken. Tylosin tartrate absorption of turkey and chicken eggs dipped using pressure and temperature differentials. Poult. Sci. 49:1641-1649. 1970.

12. Wise, D.R., M.K. Boldero and G.A. Thornton. The pathology and aetiology of turkey syndrome '65 (TS65). Res. Vet. Sci. 14:194-200. 1973.

13. Yamamoto, R., H.B. Ortmayer and R.K. Edson. Serology of Mycoplasma meleagridis. Proc. 28th Western Poult. Dis. Conf., Mar. 19-22. Davis, Calif. 1979.

14. Yamamoto, R. Mycoplasma meleagridis infection. In: Diseases of Poultry, 7th ed. M.S. Hofstad, B.W. Calnek, C.F Helmboldt, W.M. Reid, and H.W. Yoder, Jr., eds. pp. 250-260. Iowa State Univ. Press, Ames. 1978.

Table 1. Media used for Mycoplasma meleagridis isolation and identification.

I. Agar medium for plates and slopes.

Ingredients*	Per liter
Deionized distilled water	850 ml
PPLO broth powder	21 g
Yeast autolysate**	10 g
Bacto agar	12 g
Thallium acetate	0.025% (1:4000)
Penicillin	1000units/ml
Horse serum (heat-inactivated at 56 C for 30 min.)	150 ml

Dissolve the PPLO broth powder (Difco, Detroit, Michigan) and yeast autolysate (Sigma, St. Louis, Missouri) in water. Adjust pH to 7.6. Add bacto agar (Difco) and autoclave at 15 lbs for 15 min. Add thallium acetate, penicillin, and serum to the sterilized, cooled (56 C) agar. Pour plates or prepare agar slopes (overlay tubes) by dispensing approximately 1-to-1.5-ml amounts into 16 x 125-mm styrofoam-stoppered tubes and slanting the tubes at a 20 degree angle. A glass funnel facilitates dispensing the agar. The agar plates have a shelf life of about 2 weeks, and the overlay tubes may be stored for about 2 months at 4 c.

*Mycostatin (50 U/ml) may be added to inhibit growth of fungi.

**Fresh yeast extract may be substituted for the autolysate. Dissolve 250 g of dry yeast (Red Star Active Dry Yeast, Universal Foods, Milwaukee, Wisc.) in 1,000 ml distilled water. Incubate at room temperature and at 37 C for 30 min. each and then boil for 3 min. (during the extraction process, the yeast should be constantly stirred). Clarify through a filter and adjust the volume to 350 ml if necessary. Dispense and autoclave at 10 lbs. for 15 min. Add 100 ml per liter of medium (reduce distilled water in medium to 750 ml).

II. Broth for overlay tubes.

The broth is prepared in the same manner as the complete agar medium with the omission of agar. In addition, polymyxin B sulfate (100 U/ml) may be added to the broth at the time the other inhibitors are added if specimens from contaminated sites such as the phallus are to be cultured. Mycostatin (50 U/ml) may also be added to the broth. The broth is dispensed into the overlay tubes in amounts sufficient to submerge the agar slopes, about 3 ml per tube. The complete overlay enrichment medium may be stored for approximately 10 days at 4 C. Longer periods are not advised owing to deterioration of the antibiotics in the medium.

III. Differential media.

Sterile 6 mm diameter filter paper discs (Becton Dickinson, Oxnard, CA) are impregnated with 0.02 ml of 1.5% (w/v) digitonin (Sigma) in 95% ethanol (digitonin is dissolved in ethanol by heating at 56 C for 30 min.). The discs are dried overnight at 37

C and stored at 4 C. For tests, a disc is placed on an area of the mycoplasma medium heavily seeded with the unknown, and the plate is incubated at 37 C for 3-5 days. Mycoplasma sp. will show a zone of inhibition around the disc while Acholeplasma sp. will not. Include known positive and negative cultures as controls.

Glucose broth is prepared from phenol red broth (Difco) with glucose (1%), horse serum (15%), and thallium acetate and penicillin. Arginine broth is prepared similarly with the replacement of glucose with arginine free base (1%). The pH of glucose broth should be adjusted to 7.8 and that of arginine broth to 7.3 Phenol red broth without glucose or arginine is used as controls. Note that yeast is not added to these broths.

1. Transmission cycle of MM. Venereal transmission is the most important mode of MM transmission. See text for other details.
2. Airsacculitis in day-old poults. The thoracic air sacs are most commonly involved in egg-borne infections.
3. Exposed left thoracic air sac of the poult shown in slide 2. Note the yellow, purulent exudate.
4. MM-caused airsacculitis in a 4 week-old poult; the lesion has progressed from the thoracic air sacs to the abdominal air sacs by this age. If the disease is uncomplicated, the lesions will regress within 16 weeks.
5. Bowing of the tarsometatarsal bones of a 3-week-old poult infected during embryonic development with the pathogenic RY-39 strain of MM.
6. Bowing of tarsometatarsal bones and hock joint swelling in 10-

week-old poults infected naturally with MM by the egg-borne route.

7. Everted phallus of a mature turkey tom. A cotton swab (not shown) is rubbed between the phallus folds for specimen collection for MM isolation. On occasion, it may be necessary to clean the phallus of feces with cotton before sampling. Three repeat samplings just before use of the toms for milking will usually identify phallus-infected toms.
8. Everted vagina of a mature turkey hen. A cotton swab is being inserted approximately 1 inch into the vagina for specimen collection for MM isolation. This procedure is highly efficient in determining the egg-transmission status of a flock. Although the trachea is also a preferred site of MM isolation, it does not accurately reflect the egg-transmission status.
9. A flock of mature hens naturally infected in the respiratory and reproductive tracts with MM. Such a flock will show no clinical signs of infection and could not be differentiated from a MM-free flock. Infection status can be determined only by cultural and serological procedures. Similarly, male flocks show no signs of infection with MM.
10. Primary isolation technique for MM. A specimen collected on a sterile swab is placed into an enrichment overlay medium; note that the swab "handle" protrudes outside of the styrofoam plug. After 4-6 days incubation at 37 C, the swab is squeezed against the side of the tube to remove excess fluid and then

streaked on a quadrant of the marked agar plate. If samples are from a common source, 6 to 8 specimens may be plated on a single plate.

11. The inoculated plates are placed in a large-mouth, screw-capped jar with moisture added in a tube or provided by water-soaked cotton placed in a petri dish. The use of a candle to generate CO_2 is optional. On occasion, cultures with fungal contamination on primary isolation plates have been salvaged by subculture onto fresh agar and incubating under anaerobic conditions. The use of mycostatin-containing medium also should obviate such problems.
12. After 3-7 days incubation at 37 C, the plates are examined under a dissecting microscope for the typical fired-egg-shaped colonies of mycoplasma. MM colonies will vary in size from 0.04 to 0.2 mm in diameter.
13. Colonies suspected of being mycoplasma may be confirmed by the Dienes' staining technique (5). Agar block cutout with colonies (1.0 x 1.5 cm sq) is placed colony side up on a microscope slide. A Dienes-stained coverslip is placed over the agar block. Mycoplasma colonies stain blue, showing delicately structured coccoid forms within the colonies when the preparation is examined at 400 and 1,000 magnification.
14. Direct fluorescent staining of MM colonies with fluorescence-labeled rabbit anti-MM Immunoglobulin g (green). The brownish-yellow colonies are M. gallisepticum (MG) colonies stained with rhodamine-labeled rabbit anti-MG IgG. The fluorescent-antibody

technique is a definitive method for MM identification.

15. The MM rapid plate (RP)-agglutination test is still the most rapid and reliable test available for serodiagnosis of MM. A stained antigen may be purchased commercially (Salsbury Lab., Charles City, IA). Usually 10 to 12 samples are tested and read at one time. Twofold dilutions (starting from 1:5) of positive samples are retested to determine the RP test titer. A RP titer of 1:5 is considered significant, but some samples should react at dilutions of 1:10 or higher for a positive flock diagnosis.

16. The MM micro-hemagglutination-inhibition (micro-HI) test is a supplemental test used to confirm results of RP test reactions. Two or 4 units of antigen are used in the test; a titer of 1:40 is considered suspect, and 1:80 or higher is a reactor. A positive RP and a positive micro-HI is strong evidence that a flock is infected. However, a positive RP and a negative micro-HI must be interpreted more cautiously. Under such circumstances, a careful flock history and a retest 2-3 weeks later would be helpful to determine the true status of the flock. Recently vaccinated animals to erysipelas will give false-positive reactions in the RP but not in the micro-HI test.

17. The microagglutination test has the same sensitivity but greater specificity than the RP test. It is a useful primary and supplemental test. A titer of 1:20 is considered suspect, and 1:40 or greater is a reactor. Negative reactions show a button, and positive shows diffuse agglutination in the cups.
18. The micro-enzyme-linked immunosorbent assay (micro-ELISA) was recently developed for MM serodiagnosis. It has a higher sensitivity than the micro-HI and appears promising as a supplemental test. Negative reactions tend to be colorless, and positive reactions give various shades of green in the micro-cups. A 1:100 dilution of serum giving a reading of 0.2 or greater (0.D.415) is considered positive.
19. The temperature differential method of egg-dipping is shown. Eggs warmed to 37 C are dipped for several minutes in an antibiotic solution held at 4 C. Another commonly used procedure is the pressure differential method: antibiotics are incorporated into eggs by a pressure differential created by a vacuum pump. Dipping eggs by either procedure will reduce but not eliminate MM completely from groups of infected eggs.
20. The bottom-end system of treatment (BEST) is a method whereby antibiotics are incorporated into fresh eggs via the bottom end with a syringe and needle. Recently, a combination procedure of dipping and BEST injection has been used to successfully eradicate MM from breeder flocks.