Acinetobacter:

- They are Gram-negative diplococcobacilli
- Unlike *Moraxella* species, members of this genus are oxidase negative and grow well at 22^0C.
- They grow on MacConkey agar and are resistant to penicillin.
- Like *Moraxella* species, they are not fermentative.
- They are found in soil, water and sewage and as part of the normal flora of animals and humans.

Important species

- *A. calcoeceticus* and *A. Iwoffii*.

Both may be opportunistic pathogens of humans and animals. Their pathogenic roles are not well known.

HAEMOPHILUS

- Members of this genus require for propagation one or both of two growth factors: porphyrins (heme) or nicotinamide adenine dinucleotide (NAD, NADP) originally called X (heat-stable), and factor V (heat-labile), respectively.
- *Haemophilus paragallinarum* (the cause of infectious coryza in chickens) is one of the most important type species of veterinary importance.
- *H. parasuis*, (the cause of a septicemic disease called Glasser's disease or polyserositis, and seconday respiratory disease of swine), and
- *Histophilus somni* (the cause of septicemic, respiratory, and genital tract disease in cattle and sheep). *Histophilus somni* is the name now given to those micro-organisms previously knwon as "*Haemophilus somnus*" "*Haemophilus agni*", "*Histophilus ovis*."

Characteristics of the genus;

- Gram-negative coccobacilli and facultative anaerobes.
- Typically oxidase-positive (differentiating them from members of the family *Enterobacteriaceae*).
- Most are commensal parasites of animals.

Descriptive Feature

Morphology and Staining

- Though members of the genera *Haemopilus* and *Histophilus* are gram-negative rods/coccobacilli, they can sometimes form long filaments.
- Some species (*H. paragallinarum* and *H. influenza* are encapsulated.
- The genus name *Haemophilus* is inferred from the fact that these organisms require factors X and V in blood for growth. Species designated with the prefix 'para' require only V factor. On blood agar, *Haemophilus* colonies cluster around a *Staphylococcus* streak line in a phenomenon called *satellitism*.

Virulence factors

- Adhesins. allow the organisms to adhere to cells lining a particular niche, as well as to the surface of so-called "target" cells prior to the initiation of disease (in some cases, niche and target cells may be the same). This is common among members of this genus.
- Capsules. This component found on the cell wall of some bacteria is used to interfer with phagocytosis (antiphagocytic) by preventing deposition of membrane attack complexes generated by the activation of the complement system. *Haemophilus influenzae* and *H. paragallinarum* produce capsules.
- Call wall. Lipopolysaccharde (LPS) component of gram negative bacteria elicits inflammatory response when they bind to lipopolysaccharide binding protein (LBP) (a serum protein). The complex formed by LPS-LBP is transferred to the blood and combines with the blood phase of CD14. The CD14-LPS-LBP complex binds to Toll-like receptor proteins located on the surface of macrophage cells, thereby triggering the release of pro-inflammatory cytokines.
- The cell well lipopolysacharide of *Histophilus somni* is termed lipooligosaccharide (LOS). LOS under the control of the gene *lob* (for LOS biosynethesis), undergoes phase

variation resulting in the production of various epitope expression with resultant changes in the carbohydrate portions of the *LOS*.

- Iron Acquisition iron is an absolute growth requirement in bacteria existence, hence, bacteria must acquire this substance. *Haemophili* and *Histophilus* bind transferrin-iron complexes through the use of iron-regulated outer membrane proteins expressed in these bacteria when there is iron depletion in their environment (so-called transferring binding proteins, or Tbps). Iron is then acquired by these bacteria from the transferring-iron complexes bound to their surface.

Growth Characteristics

- Members of the genera *Haemophilus* and *Histophilus* are facultative anaerobes.
- Typically oxidase-positive, and attack carbohydrates fermentatively.
- Carbon dioxide enhances growth of some strains.
- Growth factors may be supplied as hemin (X factor) and NAD (V factor). A medium naturally containing them is chocolate agar, a blood agar prepared by addition of blood when the making regular blood agar). This procedure liberates NAD from cells and inactivates enzymes destructive to NAD.

Biochemical Activities

- *Haemophilus* and *Histopilus* of animals are oxidase and nitrates-positive and ferment carbohydrates.
- In non specialist laboratories, a presumptive identification of the fastidious *Haemophilus* species is based on host species, clinical signs and lesions, colonial and microscopic characteristics, X and V factor requirements, oxidase and catalase reactions and whether or not $C0_2$ enhances growth.
- *H. somnus* is variable in biochemical activities and the most reliable reactions are oxidase-positive, catalase-negative with $C0_2$ giving a considerable enhancement of growth. Indole positive reaction is usually diagnostica.

Variability

- There are three serotypes; A-C in the so-called Page scheme, or I-III in the Kume scheme) of *H. paragallinarum*
- There are at least sevenserotypes of *H. parasuis*.

Transmission

- Transmission of haemophili and *Histophilus* is by airborne or through close contact.

Laboratory diagnosis

Specimen

Specimen collection should be based on clinical disease and must be protected from desiccation as these organisms are fragile.

Refrigeration at $4^{o}C$ and use of transport media are of little relevance in the preservation of these genera. Hence, deep freezing at $-60^{o}C$ is the most reliable.

Direct microscopy

Demonstration of Gram negative rods in smear is difficult. Fluorescent antibody technique is advisable

Isolation

To culture successfully, X and V factors must be supplied for members of genus *Haemophilus* except *H. somnus*.

X factor is present in 5% blood agar while V factor is available in red cells but susceptible to NADases enzymes present in blood.

During the preparation of chocolate agar, V factor is released from the red cells, the NADases are destroyed by heat, and the heat stable X factor remains unaffected. Also, a streak of *Staphylococcus aureus* made across blood agar plate will provide V factor. V-factor requiring haemophili grow as satellite colonies around the streak.

Successful culture of many *Haemophilus* species is enhanced in an atmosphere of 10 per cent Co_2. Hence, inoculated chocolate agar plates should be incubated under 10 per cent Co_2 at $35\text{-}37^{o}C$ for 3-4 days, although some may grow in 24 hours.

Identification

Colonial morphology

Colonies may appear small and dewdrop-like after 24-48 hours of incubation and are not consistently haemolytic. A few strains of *H. somnus* may show clearing around the

colonies especially on Columbia-base sheep blood agar. *H. somnus* colonies may also appear yellowish in a loopful of growth in a confluent lawn.

Microscopic appearance

- Haemophili are small gram-negative rods that can be coccobacillary in form. More rarely short filaments occur.

Tests for X and V factor requirements

- V factor:

  The need for the V factor can be demonstrated by satellitism around V factor-producing bacterium such as *Staphylococcus aureus*. The test is carried out on tryptose agar which does not contain either the X or V factor.

- Disc Method for X and V factors:

  Three different commercial discs impregnated with V factor, X factor and XV factors, respectively, are placed on a streaked lawn of suspected bacterium inoculated on a trptose agar plate. Colonies will cluster around the disc(s) supplying the required growth factor(s). However, the results of this test can be invalidated:

a) If there is a carry-over of, particularly, the X factor from a previous richer medium.
b) If a contaminating colony is present on the plate, this may act as a feeder-organism.
c) If the test medium contains traces of X or V factors.

- Porphyrin test:

This test is used for the determination of the requirement for the X factor. A loopful of growth from a 24 hour culture is suspended in 0.5ml of a 2mM solution of delta-aminolevulinic acid (ALA) hydrochloride and 0.8mM $MgSO_4$ in 0.1 M phosphate buffer at pH 6.9. It is incubated for at least 3-4 hours at 37^0C and exposed to a wood's UV lamp in a dark room. A red fluorescence glow indicates the presence of porphyrin and suggests that X factor is not required. The test principle is based on the ability of X factor-independent strains to convert ALA, a porphyrin precursor, to porphyrin (an intermediate in the haemin biosynthetic pathway). Haemin- dependent strains lack the appropriate enzymes and cannot convert ALA to porphyrin.

Serology

- Serological techniques such as slide and tube agglutination tests, agar gel precipitation, latex agglutination and haemagglutination and haemagglutination-inhibition tests are capable of detecting antibodies to *H. paragallinarum* in poultry after 1-2 weeks of infection and in career birds.
- Although evidence abound of the presence of antibodies to *H. somnus* in cattle populations, diagnostic test for clinical cases are scarce.

BORDETELLA

- The bordetellae are small, Gram-negative rods that tend to be coccobacilliary
- They are strict aerobes and do not attack carbohydrates.
- *B. avium* and *B. bronchiseptica* are motile by peritrichous flagella but *B. pertusis* and *B. parapertusis* are non-motile. All are catalase-positive and oxidase-positive. *B.bronchiseptica* and B. avium will grow on MacConkey agar.

Natural Habitat

- The bordetellae are primarily inhabitants of the upper respiratory tract of healthy and diseased humans, animals and birds.
- *B. pertussis* and *B. parapertussis* are human pathogens causing whooping cough and mild form of whooping cough, respectively.
- *B. bronchiseptica* can be present in the upper respiratory tract of infected pigs, dogs, cats, rabbits, guines-pigs, rats, horses and possibly other animals. *B.bronchiseptica* and toxigenic *Pasteurella multocida* type D are the primary agents of swine atrophic rhinitis. *B. bronchiseptica* is also associated with kennel cough of dogs.
- *B. avium* inhabits the respiratory tract of infected poultry, majorly turkeys. The organism was formerly named *Alcaligenes faecalis.* It causes turkey coryza, a severe rhinotracheitis of young poults.
- Mode of transmission of Infection is majorly by aerosols but in turkeys indirect spread can occur via water and litter.

Laboratory Diagnosis

Specimens

- Include nasal swabs, tracheal washings and pneumonic lungs.
- In young animals and other animals with narrow nasal orifices, such as dogs and laboratory animals, the narrow gauge, flexible swabs designed for human infants (such as Mini-Tip Culturette swabs) can be adapted for use.

Direct microscopy

- bordetellae are small Gram-negative coccobacilli.
- Rather than direct smears from specimen, fluorescent antibody technique is preferrable.

Culture

- *B. avium* and *B. bronchiseptica* grow well on both sheep blood and MacConkey agars media. The plates are incubated aerobically at 37^0 C for 24-48hours.
- Selective media include;
  a. MacConkey agar with 1 per cent glucose and 20 μg/ml furaltadone or blood agar with 2 μg/ml clindamycin and 4 μg/ml neomycin.
  b. Smith- Baskerville (SB) medium is an indicator medium. This medium is very specific for the isolation of *B. bronchiseptica* from pigs. However, it can also be used is used for the isolation of strains from dogs or rabbits
  c. B.avium will grow well on SB medium, with or without the antibiotic supplement. The inoculated SB medium is incubated aerobically at 37^0 C for 48 hours.

Identification

Colonial morphology

- On sheep or horse blood agar;
  a. *B. bronchiseptica* produces very small, convex, smooth colonies with an entire edge. Some strains may be haemolytic.
  b. *B. avium* are similar but are non-haemolytic.

Phase modulation exists in both species and attributed to loss of a capsule-like structure during subculture. The phases are;

i. Phase I- This encapsulated virulent phase appear convex and shiny,

ii. Phase II appear larger, circular and convex with a smooth surface and

iii. Phase III is avirulent and colonies are large, flat and granular with an irregular edge.

- On MacConkey agar, colonies are small, pale with a pinkish hue and amber discolouration of the underlying medium. *B. avium* and *B. bronchiseptica* colonies appear very similar on MacConkey agar.
- Smith-Baskerville (SB) medium which is an indicator medium contains the pH indicator bromothymol blue and the agar is green at PH 6.8.

  i. After 24 hours' incubation, *B. avium* and *B. bronchiseptica* colonies appear small (0.5 mm diameter or less), blueish with a lighter blue (alkaline) reaction in the medium around them.

  ii. After 48 hours' incubation, the colonies increase to about 1.0-2.0 mm diameter, blue or blue with a green centre and the surrounding medium is blue. Fermentative bacterial contaminants produce acid reaction change colonies and their surrounding medium to yellow.

Biochemical reactions

- *B. bronchiseptica* is oxidase, catalase, citrate, urease and nitrate positive. It is motile and does not ferment carbohydrates
- *B. avium* and *Alcaligenes faecalis* show similar reactions to those of *B. bronchiseptica* but are negative to urea and nitrate tests.
- *A. faecalis* is a ubiquitous organism found in the soil, water and faeces. This contaminant has many similar properties with *B.avium* and can be mistaken for it.

Haemagglutination test

- *B. bronchiseptica* possesses a haemagglutinin and will haemagglutinate washed sheep erythrocytes. 24-hour culture colonies are more reliable for detecting haemaglutinin as older colonies tend to lose their haemagglutinating ability with age.
- Two colonies of a suspected *B. bronchiseptica* culture should be suspended in a drop of physiological saline on a slide. An equal volume of a 3 per cent suspension of a washed sheep red cells is added and mixture gently rocked. To rule out autoagglutination, controls should be set-up to include a suspension of colonies without erythrocytes and a

suspension of erythrocytes alone. *B. bronchiseptica* haemagglutinate the red cells within 1-2 minutes.

Serology

- Microagglutination, Tube agglutination and ELISA procedures have been developed for *B. avium* and *B. bronchiseptica.*

Animal inoculation

- *B. avium* and *B. bronchiseptica* produce dermonecrotising toxins which are intracellular, heat-labile and form part of their virulence factors. No evidence of cross-reactivity has been demonstrated between these two toxins.
- The dermonecrotising toxin of *B. bronchiseptica* has been shown to be lethal when inoculated intraperitoneally into mice and causes skin necrosis when injected intradermally into guinea-pigs. Fatal infections can also be produced in guinea-pigs by injection of young, intact cells given intraperitoneally.

Taylorella equigenitalis

- Gram-negative, facultatively anaerobic rods.
- The genus contains two species;
  T. equigenitalis, the cause of contagious equine metritis (CEM), and
  T. asinigenitalis an inhabitant of the genital tract of clinically normal male donkeys. Because of its clinicial and economic importance, *T. equigenitalis* will be discussed in detail. Because of its phenotypic similarity to *T. equigenitalis, T. asinigenitalis* will only be mentioned.

Taylorella equigenitalis (formerly *Haemophilus equigenitalis*)

- Gram-negative rod, facultative anaerobe and non-motile.
- Oxidase-positive, catalase-positive, phosphatase-positive and produces no acid from carbohydrates.
- *T. equigenitalis* is a fastidious and slow-growing. Optimal growth is obtained on chocolate agar with a rich base (Eugon or Columbia agar) at 37^0 C under 5-10 per cent CO_2.

- It does not grow on MacConkey agar.

Natural Habitat

- *T. equigenitalis* is the causal agent of contagious equine metritis (CEM).
- It resides exclusively in the equine genital tract. Stallions develop no signs of this highly contagious disease

Transmission

- Transmission is essentially veneral, but can also be by attendants and via instruments especially in mares.
- The organisms can be isolated from neonatal and virgin animals.
- *T. equigenitalis* can be found on the surface of the penis, in preputial smegma and in the urethral fossa. The infection in mares causes a temporary infertility and occasionally abortion within 60 days of pregnancy.

Laboratory diagnosis

Specimens

- Mares: cervix, uterus, clitoral fossa and clitoral sinuses.
- Stallions: urethra, urethral fossa and diverticulum, prepuce and pre-ejaculatory fluid.
- Samples can be obtained from stallion after servicing two maiden mares. The mares are then sampled instead of the stallion. The specimens are carefully collected using sterile swabs which are placed into Amies transport medium with charcoal for transportation to the laboratory not later than 48 hours.

Direct microscopy

- Gram-negative rods, coccobacilli or short-filaments
- Gram-stained smears are only useful on uterine exudates from mare suspected of clinical *T. equigenitalis.*

Isolation

- Chocolate agar with a highly nutritive base such as Eugon or Columbia agar and preferably equine blood. The inoculated plates should be incubated at 37^0 C under 10 per cent CO_2. Although growth may be seen at 48hours, negative plates should only be discarded after 7 days no growth.

- Selective media are required to suppress contaminating bacteria. If streptomycin is used as one of the selective agents, two plates should be inoculated in parallel, with and without streptomycin, as some strains of *T. equigenitalis* are susceptible to this antibiotic.

Identification

Colonial morphology

- Colonies are less than 1mm in diameter and appear, shiny, smooth and grayish-white.

Microscopic appearance

- Gram-negative pleomorphic coccobacilli are seen in smears.

Biochemical reactions

Catalase and Oxidase positive colonies with macroscopic and microscopic appearances consistent with the organism are subcultured onto Eugon chocolate agar without antibiotics and subjected to further tests:

- Inability to grow in air.
- Agglutination with *T. equigenitalis* specific antiserum in a slide test. Weak spontaneous agglutination may sometimes occur in the saline control.
- Phosphatase activity: 0.5ml of p-nitrophenyl phosphate solution (1mg/ml) is added to a suspension of the suspect colonies in 0.5 ml of Tris buffer (PH 8.0). The mixture is incubated at 37^0C for up to 2 hours. A yellow colour indicates a positive result.

Acknowledgment

I wish to acknowledge the use of the books titled “Veterinary Microbiology 2ed” and “Clinical Veterinary Microbiology”. The books are recommended for this course.

1. Veterinary Microbiology 2ed.
Dwight C. Hirsh
N. James MacLachian
Richard L. Walker

2. Clinical Veterinary Microbiology
P. J. Quinn
M. E. Carter
B. Markey
G. R. Carter