

# **VPM 401: VETERINARY BACTERIOLOGY**

**LECTURE NOTES**

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## VPM 401: VETERINARY BACTERIOLOGY COURSE OUTLINE

<b>Topics:</b>	<b>Lecturer</b>
<i>Dermatophilu</i>	M. A. Oyekunle
<i>Actinobacillus</i>	M. A. Oyekunle
<i>Nocardia</i>	M. A. Oyekunle
<i>Mycobacterium</i>	M. A. Oyekunle
<i>Mycoplasma</i>	M. A. Oyekunle
<i>Fusobacterium</i>	M. A. Oyekunle
<i>Bacteroides</i>	M. A. Oyekunle
<i>Dichelobacter</i>	M. A. Oyekunle
<i>Pasteurella</i>	M. A. Oyekunle
<i>Manhaemia</i>	M. A. Oyekunle
<i>Francisella</i>	M. A. Oyekunle
<i>Yersinia</i>	M. A. Oyekunle
Bacterial pathogens of fish	M. A. Oyekunle
<i>Staphylococcus</i>	O. E. Ojo
<i>Streptococcus</i>	O. E. Ojo
<i>Bacillus</i>	O. E. Ojo
<i>Clostridium</i>	O. E. Ojo
<i>Listeria</i>	O. E. Ojo
<i>Erysipelothrix</i>	O. E. Ojo
<i>Corynebacterium</i>	O. E. Ojo
<i>Actinomyces</i>	O. E. Ojo
<i>Rodococcus</i>	O. E. Ojo
<i>Escherichia coli</i>	O. E. Ojo
<i>Klebsiella</i>	O. E. Ojo
<i>Enterobacter</i>	O. E. Ojo
<i>Citrobacter</i>	O. E. Ojo
<i>Salmonella</i>	O. E. Ojo
<i>Shigella</i>	O. E. Ojo
<i>Proteus</i>	O. E. Ojo
<i>Morganella</i>	O. E. Ojo
<i>Providencia</i>	O. E. Ojo
<i>Pseudomonas</i>	O. E. Ojo
<i>Acinetobacter</i>	M. Agbaje
<i>Neisseria</i>	M. Agbaje
<i>Moraxella</i>	M. Agbaje
<i>Brucellosis</i>	M. Agbaje
<i>Bordetella</i>	M. Agbaje
<i>Haemophilus</i>	M. Agbaje
<i>Vibrio</i>	M. Agbaje
<i>Campylobacter</i>	M. Agbaje
<i>Helicobacter</i>	M. Agbaje
<i>Leptospira</i>	M. Agbaje
<i>Borrelia</i>	M. Agbaje
<i>Brachyspira</i>	M. Agbaje
<i>Treponema</i>	M. Agbaje
<i>Taylorella</i>	M. Agbaje
Microbiology of water, food and milk	M. Agbaje

# VPM 401 – VETERINARY BACTERIOLOGY

DR. M. A. OYEKUNLE

## Introduction

### What is bacteriology?

Bacteriology is the study of bacteria

### Why do we study bacteria?

We study bacteria in Veterinary Medicine or medicine because bacterial diseases are among the most important and common problems that animal and fish keepers/managers must deal with. Therefore, the veterinarian must be equipped to know about these organisms. Because infections frequently involve more than one system, veterinary microbiologist/bacteriologists have generally resisted the systemic approach to teaching infection diseases.

However, student may develop tables to assist himself in system orientation to infections agents.

We study these organisms to know which disease they are involved so as to find a treatment. Therefore, the approach to their study will include knowing fully about them.e.g. Their

- History
- Habitat
- Characteristics – Colonial/Culture characteristics
  - Cell morphology
  - Staining characteristics
  - Biochemical characteristics
  - Genetic characteristics
- Among others

### The Actinomycetes

- They consist of a group of filamentous microorganisms occupying an intermediate position between bacteria and fungi.
- Their identity as bacteria was confirmed by:
  - \* Their prokaryotic cellular organization
  - \* Their cell wall chemistry
  - \* Their nitrogen metabolism
  - \* Their sensitivity to antibiotics and phages.
- There are two major groups of actinomycetes
  - \* Aerobic actinomycetes
  - \* Anaerobic actinomycetes
- They cause infections in animals and humans.
- There are also a large number of nonpathogenic species

#### 1. Genus *Actinomyces*

- Are pleomorphic Gram Positive coccobacilli, rods, filament, branching or non branching cells.
- Non-motile, non-spore forming.
- membrane of the oral and nasal cavities and the genital tract.
- Important species are:
 

A. Israeli	human actinomycosis	obligate anaerobic
A. bovis	cattle actinomycosis	obligate anaerobic
A. viscosus	Dog periodontal disease	facultative anaerobic
A. hordeovulnesis	Human chronic suppurative Dog infection	facultative anaerobic
A. naeslundii	Human Periodontal infection Dental caries	facultative anaerobic
A. pyogenes	Animals Pyogenic infection	facultative Anaerobic

### **Disease- Actinomycosis**

- **Laboratory diagnosis (Actinomycosis)**
  - Direct examination
    - Small amount of pus placed in petridish.
    - This is washed with water to expose small sulphur granules.
    - Transfer granule to a slide, add a drop of 10% NaOH, add cover slip and crush by gentle pressure.
    - Characteristic ray-fungi is seen with club shaped margins under low power if actinomycosis.
    - Then remove cover slip, spread and stained by Grams.
    - If Actinomycosis, branching Gram positive filaments are observed.
- **Isolation and Cultivation**
  - Can be cultured on blood agar, brain heart infusion agar and thoglycollate broth.
  - An atmosphere containing 5-10% Co<sub>2</sub> preferred for incubation.
  - Colonies are white, rough, nodular and adhere tenaciously to the medium and difficult to remove.
  - Gram stained smears from growth on media revealed masses of Gram positive rod and slightly branched filaments.
- **Identification**
  - Based on characteristic sulphur granules
  - Demonstration of gram positive filaments
- **Treatment**
  - Drainage and antibiotic therapy

## **2. Genus *Nocardia***

- Non-motile, nonspore forming, gram positive rods which sometimes show branching.
- Partially acid fast, aerobic.
- Spits sugars by oxidation.
- Are important part of the soil and water flora.
- A number of the members of the genus cause a variety of diseases in both normal and immunocompromised humans and animals.
- Mechanisms of pathogenesis complex and not well understood but include the capacity to evade or neutralize the myriad of antimicrobial activities of the host.
- More than 40 species have been described.
- Important species include

<i>N. asteroides</i>	-	Human and animals
<i>N. brasiliensis</i>	-	Human
<i>N. caviae</i>	-	Human, bovine mastitis, guinea pig
<i>N. farcinica</i>	-	Cattle

#### **Mode of Infection**

- By inhalation, through wounds, hands and feet of laboratory workers.
- Usually exogenous

#### **Laboratory diagnosis**

##### **Direct examination**

- Gram stained smears of pus/lesions reveal Gram positive branching filaments with or without clubs.
- Stains partially with ZN stain.
- Giemsa stain can also be used.

Experimental animal: guinea pig susceptible

##### **Isolation and Cultivation**

- Organism grows on blood agar or any other enriched media.
- The colour of the colony varies from chalk white to deep orange.

##### **Identification**

- Based on demonstration of typical organism, colonial, cultural and morphological characteristics.

##### **Treatment**

- Various drugs useful including sulphonamides and antibiotics.

### **3. Dermatophilus congolensis**

- Gram positive branching filamentous rods, aerobic and nonspore forming, non acid fast.
- Produce motile zoospores.
- Unique medically because natural growth cycle is restricted to the living layer of the epidemics of animal and human skin.

Pathogenicity: Causes dermatophilosis in cattle and dermatophilus infection in other animals which is characterized by scabs formation on the skin.

### **Laboratory diagnosis**

Specimen- Infected Scab

#### **Direct Examination**

- Many procedures employed in making impression smears.
- But better if impression smear is made from the moist concave undersurface of freshly removed scabs.
- Stains well with dilute carbol fuchsin or methylene blue stain, Gram stain or preferably 1:10 dilution of Giemsa stain for 30 minutes.

#### **Isolation and Cultivation**

Organism grows well on media containing blood or blood product.

- Colony : - Small, rough, graywhite colonies appear in 24-48 hours of incubation.
- Colonies are yellowish to orange.
  - Produces B haemolysis on sheep or horse blood agar. On human blood, haemolysis is narrow and hazy.
  - Motile zoospores are formed as a result of the septation of hyphal element
  - Zoospores possess polar flagella.
  - Gram positive, branching hyphal elements in various stages of segmentation are seen.
  - Two colony forms can be demonstrated.
  - (i) Rough - grows into the agar and difficult to remove and emulsify in water or saline.
  - (ii) Smooth- easy to remove from plate and emulsify in water or saline.

#### **Antigenic Components**

- Five (5) antigenic types demonstrated using agar gel precipitating test.

#### **Treatment**

Use of various drugs, chemicals and concoitious are in practice.

#### **4. *Mycobacterium***

- Are Gram positive (Not easily stained by Gram method acid fast, small rods, non-motile.
- Filamentous and branching forms occur.

- They don't stain readily, but when they do so stain with basic dyes.
- They resist decolourization by acid.
- There are more than 50 Mycobacteria species including many that are saprophytes.

i. Runyon Classification of Mycobacteria (Runyon's group)

<b>Classification</b>	<b>Organisms</b>
Tuberculosis complex	<i>M. tuberculosis</i> <i>M. bovis</i> <i>M. africanum</i>
Photochromogens  (Produce pigment in light)	<i>M. asiaticum</i> <i>M. kansasii</i> <i>M. mageritense</i> <i>M. goodii</i>
Scotochromogens  (produce pigment in the dark)	<i>M. flavescens</i> <i>M. goodii</i> <i>M. scrofulaceum</i> <i>M. szulgai</i>
Non-chromogens  (No pigment produced)	<i>M. avium complex</i> <i>M. celatum</i> <i>M. haemophilum</i> <i>M. gastri</i> <i>M. genovense</i> <i>M. malva</i>

ii. **Rate of growth**

- Rapid growers
- Slow growers

iii. **Anonymous mycobacteria**

Are atypical unclassified mycobacteria that have been recovered from animals and man.

iv. **Saprophytic or non pathogenic mycobacteria**

Mycobacteria considered to be non pathogenic or not previously identified are now becoming epidemiologically important particularly in the AIDS era because of their high resistance to antibacterial agents.

v. **New species**

**Laboratory diagnosis of tuberculosis**

- Based on
  - (i) Microscopy
  - (ii) Culture
  - (iii) Immunological test
  - (iv) Molecular characterization

(v) Others

Specimen: Different samples may be used depending on the clinical picture of the disease.

**5. Genus *Actinobacillus***

- Gram negative, small rod, non-motile, non-spore forming, aerobic and fermentative.
- Rarely grows in filaments, and if so, filaments show some branching.
- Has tendency for bipolar staining

Important species include

*A. pleuropneumoniae* - pig

*A. equulin-* Horse (fals) and occasionally pig  
- joint illness, navel illness

*A. suis* – pig

*A. seminis* – sheep (ram) – affecting ram epididymus

- Natural infections with *A. liquieresii* occur in both cattle and sheep and are characterized by infections granulous containing pus affecting the soft tissue in the region of the head e.g. tongue.

**Laboratory diagnosis**

Granula/Pus specimen examined in the same manner as in actinomycosis.

- Small gram negative rods demonstrated in the lesion.

**Isolation and Identification**

- Specimen
- Pus or necrotic material from early lesions.
  - Natural seeded on blood or serum agar.
  - Incubated at 37°C under 10% CO<sub>2</sub> accelerated growth.
  - Subcultured strains grow well in air.
  - In media contained fermentable carbohydrate long almost filamentous form are seen.
- Colonies
- may be mucoid or stringy when freshly isolated.
  - can be white, grayish-white, yellowish or bluish in colour.
- Cultural
- Are aerobic to facultative anaerobic
- Characteristics
- Are micro aerophilic on primary isolation.
- Biochemical characteristics – Acid but no gas from carbohydrate when fermented.
- Pathogenicity
- Pathogenic to animals.  
Some species can affect humans disease produced by *A. liquieresii* can be similar to that produced by *Actinomyces* and *M. haemolyticus*.

## Differentiation of the species of the Genus *Actinobacillus* using biochemical characteristic

<i>A. liquieresii</i>	+	+	+	-	+	-	+
<i>A. Equuli</i>	-	-	+	-	+	+	+
<i>A. Seminis</i>				-	-	-	-
<i>M. haemolyticus</i>	+	+	+	-	+	-	+

### 6. Genus *Mycoplasma*

- Are bacteria
- Members of the genus are characterized by the absence of a cell wall.
- They are pliable and can pass through the pores of filters that retain bacteria.
- Most members have sterol in their membrane which provides added strength and rigidity protecting the cells from osmotic lysis.
- They are among the smallest form of life.
- Their genomes are thought to be the minimum size for encoding the essential functions for a free living organisms.
- Are facultative anaerobic or obligate anaerobic.
- Are pleomorphic.
- Because they have cell membrane, RNA and DNA, they differ from viruses.
- *Mycoplasma* can resemble fungi because some produce filaments that are commonly seen in fungi.
- It is because of these filaments that scientists named them *mycoplasma* i.e. *myco* means “fungus”.
- They stain poorly, but Giemsa can be used to demonstrate it in tissues.
- Many are unable to move because they lack flagella but some can glide.

### Cultivation and Cultural features

- *Mycoplasmas* have low biosynthetic ability.
- Therefore they need rich medium containing natural animal protein (blood serum) and in most cases sterol compounds.
- *Mycoplasma* colonies on solid media produce a characteristic “fried egg” appearance.

### Cell morphology

- Coccobacilli, cocco forms, ring forms, spiral and filaments seen in smears stain poorly, but, Giemsa can be used.
- Size 50-60 to 100-250 nm, diameter 0.3-0.8µm.
- Parasitic *mycoplasmas* contain 10-20% lipid, relatively low content of nucleic acid compared to other bacteria.
- May grow in chicken embryo.

### Viruses and Plasmids of *Mycoplasmas*

- 14 viruses identified to infect *mycoplasma*
- 6 in *Acholeplasma*
- 4 in *Mycoplasma*
- 4 in *Spiroplasma*

- There is evidence of integration of viral genomes into mycoplasma chromosomes.
- Release of virus is continuous and not accompanied by cell lysis.
- Plasmids detected in Mycoplasma, Acholeplasma and Spiroplasma.
- Acholeplasmataceae – does not depend on sterol for growth.
- Anaeroplasmataceae – strict anaerobes

## The Bacteriodes

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## BACTERIAL PATHOGENICITY

- Majority of bacteria are non-pathogenic saprophytes
- Bacteria which causes disease in humans and animals are small in number compared to those that do not cause disease
- Bacteria that cause disease are said to be pathogenic
- The development and severity of bacterial infections are influenced by host-related determinants such as phy-biological status and immune competence
- Commensal bacteria can cause opportunistic infection in the host

Steps in bacterial infection

Route of bacterial entrance into the host: skin, mucus, membranes, teat canal and umbilicus

Steps in Bacterial Pathogenesis:

Adhesion to the host cells

Local proliferation or multiplication

Damage to the host tissue

Invasion

Dissemination

Tissue and host specificity

- Virulence of bacteria relates to the ability to invade and produce disease in a normal animal
- Ability to adhere: virulent pathogens often possess specific surface molecule which allow adherence to receptors on host cells
- Adherence factors include: adhesions, fimbriae, intimin, invasion (all in gram-negative bacteria)
- Adherence factors in gram-positive bacteria: protein F (a fibrinectin-binding protein) is necessary for adherence of streptococci to respiratory epithelial-the coagulase of pathogenic staphylococci promotes adherence to fibrinogen-coated surfaces

- Capsule-like material in *Klebsiella pneumoniae* enhance its interaction with human intestinal cells
- In general, capsule are thought to hinder bacterial adherence to host cells
- Iron is essential for bacterial respiration
- Most iron in the animal host is bound by iron-binding proteins like lactoferrin and transferrin, and therefore unavailable for the bacteria
- Pathogenic bacteria obtain iron from the host by producing iron-chelating compounds like siderophores which can remove iron from transferrin and lactoferrin
- Other lyse erythrocytes to obtain iron from haemoglobin
- Bacterial multiplication, tissue invasion and avoidance of host defence mechanism

#### Mechanism employed by bacteria for survival in the host

- O antigen polysaccharide chain: length of polysaccharide chain hinders binding of the membrane attack complex of complement to the outer membrane of many gram-negative bacteria
- Capsular antigen: incorporation of sialic acid by some gram-negative bacteria has an inhibitory effect on complement activity
- Capsule production: antiphagocytic
- H-protein production: antiphagocytic activity e.g. *S. equi*
- Production of Fc-binding proteins: *Staphylococci* and *Streptococci* produce protein which bind to the Fc region of IgG and prevent interaction with the Fc receptor on membranes of phagocytes
- Production of leukotoxins: cytolysis of phagocytes by toxins produced by *Manheimia haemolytica*, *Actinobacillus species* and other pathogenic bacteria
- Interference with phagosome-lysosome fusion, allows the survival of pathogenic mycobacterium within phagocytes
- Escape from phagosomes: survival mechanism used by *Listeria monocytogenes* and *Rickettsiae*
- Resistance to oxidative damage: allows the survival of *Salmonella* and *Brucellae* within phagocytes

- Antigenic mimicry of the host antigens: adaptation of surface antigens by *Mycoplasma spp* to avoid recognition by the immune system
- Antigenic variation of surface antigens: permits survival of *Mycoplasma spp* and *Borreliae* despite the host's immune response to these pathogens
- Coagulase production: conversion of fibrinogen to fibrin by *Staphylococcus aureus* can isolate site of infection from effective immune response

Dissemination of bacteria in the host

- Avoidance of host defence mechanism is essential for successful invasion and dissemination of the pathogen
- Enzymes such as collagenases, lipases, hyaluronidases and fibrinolysin produced by bacterial pathogens facilitate breakdown of host tissue
- Bacteraemia is the transient presence of the bacteria in the blood stream without replicating
- Septicaemia is the persistent presence of bacteria multiplying in the blood stream

Damage to host tissue and associated clinical signs

- Direct damage is caused by exotoxin and endotoxin production
- Indirect damage results from the activity of enzymes secreted by the bacteria and host immune response to infection

**Comparison of exotoxins and endotoxins**

Exotoxin	Endotoxin
Produced by live bacteria	Released during death and lysis of cells
Secreted actively	Component part of cell wall
Produced by both gram-positive and gram-negative bacteria	Produced by gram-negative bacteria
High molecular weight protein	Lipopolysaccharide complex containing lipid A, the toxic component
Heat-labile	Heat-stable
Potent toxins, usually with specific activity	Toxin with moderate non-specific

	generalised activity
Not pyrogenic	Potent pyrogens
Highly antigenic	Weakly antigenic
Readily converted to toxoid	Not amenable to toxoid production
Induced neutralizing antibodies	Neutralising antibodies not associated with natural exposure
Synthesis determined extra-chromosomally	Encoded by chromosome

## ***STAPHYLOCOCCUS SPECIES***

- Gram-positive bacteria
  - Spherical (cocci) in shape
  - About 1  $\mu\text{m}$  in diameter
  - Occur in irregular clusters
  - Staphyle=bunch of grapes
  - Kokkos=berry
  - Common commensals on skin and mucous membrane
  - Often cause pyogenic infections
  - Oxidase-negative, catalyse-positive, non-motile, non-sporing facultative anaerobes
  - Important animal pathogens include *S. aureus*, *S. intermedius*, *S. hyicus*
  - Pathogenic species often produce coagulase
  - *S. aureus* and *S. intermedius* are coagulase positive while *S. hyicus* is coagulase variable
  - Coagulase negative staphylococcus are of low virulence but may occasionally cause disease in animals and man
- ▶ Diseases in animals
- Exudative epidermitis in piglets (greasy-pig diseases): *S. hyicus*
  - Tick pyaemia of lambs : *S. aureus*
  - Bovine staphylococcal mastitis: *S. aureus*
  - Botryomycosis (Scirrhus cord) (horse, pigs, cattle): *S. aureus*

- Wound infection (most animals): *S. aureus*, *S. hyicus*, *S. intermedius*
- Mastitis: *S. aureus*, *S. hyicus*, *S. intermedius*
- Bumble foot, omphalitis in poultry: *S. aureus*
- Pyoderma, otitis externa, cystitis, endometritis in dogs: *S. intermedius*

#### ▶ Diagnosis

- Sample collection: pus, exudates
- Media: grow on non-enriched media
  - Nutrient agar, blood agar
- Selective medium: mannitol salt agar (staphylococci can tolerate high concentration of NaCl). Mannitol salt agar contains 7-10% NaCl
- P- agar for cultural differentiation of staphylococci

#### ▶ Colonial characteristics

- Colour: usually white, opaque and up to 4mm in diameter. Colonies of bovine and human strains of *S. aureus* are golden yellow. Saprophytic staphylococcus may be pigmented
- Staphylococcus may produce haemolysis on sheep/ox blood agar. Types of haemolysis: alpha, beta, gamma, delta haemolysis
- *S. aureus* and *S. intermedius* produce double zones of narrow complete and wide incomplete haemolysis on blood agar
- *S. hyicus* is non-haemolytic

- ▶ Coagulase test: mix a suspension of staphylococcus isolate with rabbit plasma either on a slide or in a small tube. Coagulase convert fibrinogen to fibrin (strand/lumps)

- Slide coagulase test detects the presence of bound coagulase or clumping factor within 1 to 2 minutes
- Tube coagulase test detects free coagulase or staphylocoagulase secreted by bacteria. It is the definitive test for coagulase. The plasma clots within 24 hours of incubation at 37°C

► Differentiation tests

Differentiation of Gram-positive cocci

Organism	Appearance of stained smear	Coagulase	Catalase	Oxidase	O-F test	Bacitracin test
<i>Staphylococcus spp</i>	Irregular cluster	±	+	-	F	Resistant
<i>Micrococcus spp</i>	Packets of four	-	+	+	O	Susceptible
<i>Streptococcus and enterococcus spp</i>	Chain	-	-	-	F	Resistant

Purple agar: contains indicator-bromocresol purple, sugar (1% maltose)

Species	Colony colour	Haemolysis on sheep BA	Tube coagulase	Slide coagulase	Acetone production	Maltose utilization
<i>S. aureus</i>	Golden yellow	+	+	+	+	+
<i>S. intermedius</i>	White	+	+	V	-	±
<i>S. hyicus</i>	White	-	V	-	-	-

- Molecular procedure carried out in research and reference laboratories

Treatment: Penicillin and its derivatives

### ***STREPTOCOCCUS SPECIES***

- Gram-positive bacteria chain 1.0  $\mu\text{m}$  in diameter
- Non-motile, non-sporing, oxidase negative, catalase-negative, facultative anaerobes
- Fastidious organism requiring enriched media for growth
- Pathogenic species cause suppurative conditions such as mastitis, metritis, polyarthritits and meningitis in animals
- *Enterococcus spp* are opportunistic enteric *streptococci* found in intestinal tracts of animals and humans
- Unlike *Streptococcus spp*, *enterococci* can tolerate bile salt and therefore grow on MacConkey agar as red pinpoint colonies. Some streptococci are also motile
- Most *streptococci spp* live as commensals on the mucosae of the upper respiratory tract and lower urogenital tract
- *Streptococci* are fragile and susceptible to desiccation

#### Diseases:

- Bovine streptococcal mastitis:
  - i. *S. agalactiae*, B,  $\beta$  ( $\alpha, \gamma$ )
  - ii. *S. dysgalactiae* C,  $\alpha$  ( $\beta, \gamma$ )
  - iii. *S. uberis* NA  $\alpha$  ( $\gamma$ )
  - iv. *Enterococcus faecalis* D,  $\alpha$  ( $\beta, \gamma$ )
  - v. *S. pyogenes* A,  $\beta$

vi. *S. zooepidemicus* C,  $\beta$

- (i-iii): principal pathogens of mastitis
- (iv-vi) are less associated with mastitis

▪ Strangles in horses: *S. equi* C,  $\beta$

- Abscess and other suppurative conditions and septicaemia in many species of animals

*S. pyogenes* (A,  $\beta$ ): humans

*S. canis* (G,  $\beta$ ): dogs

*S. suis* (D,  $\alpha$  ( $\beta$ )): pigs

*S. equisimilis* (C,  $\beta$ ): horses

### Diagnosis

- History, clinical signs and pathology may be indicative of streptococcal infection
- Samples are collected and cultured promptly: streptococci are highly susceptible to desiccation. Samples include pus and exudates
- Samples can be placed in transport medium
- Stained smear of clinical samples may reveal gram-positive cocci in chains
- Samples should be cultured on blood agar and MacConkey agar
- Incubate agar plates aerobically at 37°C for 24-48 hours
- Streptococcal colonies are small, translucent and some may be mucoid
- Differentiation of the streptococci:
  - i. Type of haemolysis
  - ii. Lancefield grouping
  - iii. Biochemical testing

i. Type of haemolysis on blood agar

- Beta-haemolysis: complete haemolysis of clear zones around colonies
- Alpha-haemolysis: partial, incomplete haemolysis, greenish or hazy zones around colonies
- Gamma-haemolysis: no observable changes in the blood agar around colonies

ii. Lancefield grouping: a serological method of classification based on the group-specific C-substance. Lancefield grouping test methods include:

- Ring specification test

- Extract C-substance by acid or heat from the *Streptococcus spp*
- The extract (antigen) is layered over antisera of different specificities in narrow tubes placed in plasticine on slide
- A positive reaction is indicated by the formation of a white ring of precipitate close to the interface of the two fluids within 30 minutes

- Latex agglutination test: latex-coated group-specific antibodies are commercially available for the test. Antigen is extracted enzymatically from the *streptococcus spp* under test

- Mix antiserum and antigen together on a slide
- Positive reaction is indicated by agglutination

iii. Biochemical tests: oxidase, catalase, sugar fermentation tests. Biochemical tests are available commercially for rapid identification of streptococci

**Biochemical differentiation of equine group C Streptococci**

Species	Trehalose	Sorbitol	Lactose	Maltose
<i>S. equi</i>	-	-	-	+
<i>S. zooepidemicus</i>	-	+	+	+(-)

<i>S. equisimilis</i>	+	-	V	+
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### Differentiation of streptococci associated with mastitis:

*S. pyogenes*: Bacitracin sensitive (all group A)

*S. agalactiae*: CAMP test (Christie, Atkins, Munch and Petersen) positive with *S. aureus* and *Corynebacterium pseudotuberculosis* (all group B *Streptococci*)

*S. uberis*: Aesculin hydrolysis (black brown zones of discolouration around dark coloured colonies on Edward's medium).

*S. pneumoniae*: quellung reaction/capsule swelling test, bile solubility, Optorchin-sensitivity, *S. pneumoniae* appears a lancet-shaped organisms in pairs. It is capsulated

CAMP test: enhanced haemolysis (synergism) of staphylococcal beta-toxin or corynebacterial phospholipase D

Optorchin: ethylhydrocupreine hydrochloride

### ***LISTERIA SPECIES***

- Gram positive coccobacillary rods about 2µm in length
- Catalase positive, oxidase negative, facultative anaerobes
- There are six species in the genus, three of which are pathogenic
- *L. monocytogenes* is the most important species. It was first isolated from rabbits with septicaemia and monocytosis
- Can tolerate wide temperature (4°C – 45°C) and pH (5.5 – 9.6) ranges

#### Diseases

i. *Listeria monocytogenes*

Cattle, sheep, goats: encephalitis (neural form), abortion, septicaemia, endophthamitis.

Cattle: mastitis (rare).

Dogs, Cats, horses: abortion, encephalitis (rare)

Pigs: abortion, septicaemia, encephalitis

Birds: septicaemia

ii. *L. ivanovii*

Sheep, cattle: abortion

iii. *L. innocua*

sheep: meningoencephalitis

#### Diagnosis: (microbiological)

- Sample collection: cerebrospinal fluid, tissue from brain (medulla and pons), specimen from abortion cases: cotyledons, foetal abomasal contents, uterine discharges.  
Septicaemia: spleen, blood. Collect only fresh samples
- Smear from cotyledon or liver lesion may reveal several gram-positive coccobacillary bacteria
- Immunoflourescence using monoclonal antibodies gives rapid result

- Isolation
  - Inoculate sample onto blood agar, selective blood agar and MacConkey
  - Incubate aerobically at 37°C for 24 to 48 hours
  - A cold enrichment procedure may be necessary for recovery of *Listeria* from clinical specimen
  - Inoculate a 10% suspension of sample into nutrient/enrichment broth
  - Keep the inoculated broth at 4°C in a refrigerator
  - Subculture weekly from the broth onto blood agar for up to 12 weeks
- Two forms are formed on culture media; smooth and rough forms
  - Smooth: small, smooth, flat, more common (short filament and coccal forms, older culture)
  - Rough: young culture, entirely of long filament
- *L. monocytogenes* colonies are small, smooth and flat
- *L. monocytogenes* produces a blue-green colour with oblique illumination
- Colonies are surrounded by a narrow zone of complete haemolysis
- It is catalase positive. *Streptococci* and *Arcanobacterium pyogenes* have similar colonies but are catalase negative
- It is CAMP test positive with *Staphylococcus aureus* but not with *Rhodococcus equi*
- It hydrolyses aesculin
- Produces a characteristic tumbling motility after incubation in broth at 25°C for 2-4 hours
- Pathogenicity test in rabbit to confirm virulence: instil a broth culture into rabbit eye. Virulence strains induce keratoconjunctivitis. This is called Anton test
- *Listeria spp* are zoonotic

**Laboratory methods for differentiating *Listeria* species**

<i>Listeria spp</i>	Haemolysis on sheep blood agar	CAMP test		Acid production from sugar		
		<i>S. aureus</i>	<i>R. equi</i>	D-mannitol	L-rhamnose	D-xylose
<i>L. monocytogenes</i>	+	+	-	-	+	-
<i>L. ivanovii</i>	++	-	+	-	-	+

<i>L. innocua</i>	-	-	-	-	V	-
<i>L. seeligeri</i>	+	+	-	-	-	+
<i>L. welshimeri</i>	-	-	-	-	V	+
<i>L. grayi</i>	-	-	-	+	V	-

+ = positive reaction,                    - = negative reaction                    V = variable reaction

### ***ERYSIPELOTHRIX SPECIES/ E. rhusiopathiae***

- Gram positive slender rods which may be curved or straight
- Have tendency to form elongated filaments
- May appear in pairs or in groups
- Some thickened filaments are beaded with gram's staining
- Small rods (small form), filament (rough form)
- Both forms occur on culture media
- Smooth forms are isolated from acute infections
- Isolate from chronically infected animals from rough colonies
- Produce small colonies with incomplete haemolysis in 48 hours
- Grow over wide temperature and pH ranges
- Catalase negative
- Coagulase positive
- Non-motile, oxidase negative, facultative anaerobe
- Form H<sub>2</sub>S along slab line in Triple Sugar Iron agar

#### Diseases

- *Erysipelothrix rhusiopathiae*
  - Pigs (swine erysipelas): septicaemia, diamond skin lesions, chronic arthritis, chronic valvular endocarditis, abortion. Almost 50% of healthy pigs harbour *E. rhusiopathiae* in tonsillar tissues
  - Sheep: polyarthritis in lambs, post dipping lameness, pneumonia, valvular endocarditis
  - Turkey (turkey erysipelas): septicaemia, arthritis, valvular endocarditis

## Diagnosis

- Specimen: blood, liver, spleen, heart valves, synovial tissue. Organism rarely recovered from skin lesions or chronically affected joints
- Microscopic examination of specimen from acutely affected animals may reveal slender gram-positive rods
- Filamentous elements may be seen in samples of chronic valvular lesion
- Inoculate specimen into blood and MacConkey agar plates
- Incubate aerobically at 37°C for 24 to 48 hours
- Selective media containing either sodium azide (0.1%) or crystal violet (0.001%) may be used for contaminated samples
- Non-haemolytic, pin-point colonies appear after incubation for 24 hours and after 48 hours, a narrow zone of greenish, incomplete haemolysis develops around the colonies
- Catalase-negative, coagulase-positive (as in staphylococcus), H<sub>2</sub>S positive
- Serotyping for epidemiological studies
  - Virulence testing in laboratory animals. Because *E. rhusiopathiae* isolates vary in virulence, it is necessary to confirm virulence by intraperitoneal inoculation of mice or pigeons.
  - PCR for virulence detection

### ***CORYNEBACTERIUM SPECIES***

- Small Gram-positive pleomorphic (coccoid, club and rod forms) bacteria
- Stained smear reveals cells in palisades of parallel and angular clusters resembling Chinese letters
- Non-motile facultative anaerobes
- Catalase-positive, oxidase-negative
- Fastidious, require enrichment for growth
- Cause pyogenic infection
- Most pathogenic species are host specific
- Type species: *C. diphtheriae*, causes diphtheria in children

#### Diseases

- i. *Corynebacterium bovis*  
Host (cattle): subclinical mastitis
- ii. *C. kutscheri*  
Host (laboratory rodents): superficial abscesses, causes purulent foci in liver, lungs and lymph nodes
- iii. *C. pseudotuberculosis* (non-nitrate-reducing biotype)  
Host (Sheep and goats): caseous lymphadenitis
- iv. *C. pseudotuberculosis* (nitrate-reducing biotype)  
Host (horses, cattle): ulcerative lymphagitis, abscesses
- v. *C. renale* (type I)  
Cattle: cystitis, pyelonephritis  
Sheep and goats: ulcerative (enzootic) balanoposthitis
- vi. *C. pilosum* (renale type II)  
Cattle: cystitis, pyelonephritis
- vii. *C. cystitides* (renale type III)

Cattle: severe cystitis, rarely pyelonephritis

viii. *C. ulcerans*

Cattle: mastitis

### Diagnosis

- Specimen: pus, exudates, tissue, sample, mid-stream urine
- Direct microscopy of Gram-stained smear may reveal coryneform bacteria
- Inoculate sample onto blood agar, selective media (McLeod's blood agar, Loeffler's medium) containing potassium tellurite, and MacConkey agar
- Incubate aerobically at 37°C for 24 to 48 hours
- Identification: no growth on MacConkey agar
- Colonial Characteristics:
  - *C. bovis*: a lipophilic bacterium. Small white, dry, non-haemolytic colonies
  - *C. kutscheri*: whitish colonies, occasionally haemolytic
  - *C. pseudotuberculosis*: small whitish colonies, surrounded by a narrow zone of complete haemolysis evident after 72 hours of incubation. Colonies become dry, crumbly and cream-coloured with age
  - Members of *C. renale* group produce small, non-haemolytic colonies after 24 hours incubation. Produce pigment after 48 hours of incubation
- Microscopy: Gram's staining and Albert's staining techniques  
Albert's staining demonstrate metachromatic granule (inclusions)
- Biochemical tests
  - Nitrate reduction: *C. pseudotuberculosis* biotype
  - All pathogenic corynebacteria are urease positive except *C. bovis*

#### **Differentiation of *C. renale* group**

Feature	<i>C. renale</i> (type I)	<i>C. pilosum</i> (type II)	<i>C. cystidis</i> (type III)
Colour of colony	Pale yellow	Yellow	White
Growth in broth at pH 5.4	+	-	-
Nitrate reduction	-	+	-
Acid from xylose	-	-	+

Acid from starch	-	+	+
Casein digestion	+	-	-
Hydrolysis of Tween 80	-	-	+

- Enhanced haemolysis by *C. pseudotuberculosis* when inoculated across a streak of *Rhodococcus equi*

### ***ACTINOMYCES ARCANOBACTERIUM AND ACTINOBACULUM SPECIES***

- Gram-positive bacteria
- Require enriched media for growth
- Non-motile, non-sporing
- Morphologically heterogenous
- Anaerobic or facultative anaerobic
- Modified Z-N staining negative
- Some members have undergone changes in nomenclature
  - *Corynebacterium pyogenes* = *Actinomyces pyogenes* = *Arcanobacterium pyogenes*
- *Actinomyces species* have long filamentous morphology, although short V, Y, and T configuration also occur
- *Arcanobacterium* and *Actinobacterium* both have a coryneform morphology

#### Diseases

- *Arcanobacterium pyogenes*  
Host: cattle, sheep, pigs  
Conditions: Abscessation, mastitis, suppurative pneumonia, endometritis, pyometra, arthritis, umbilical infections
- *Actinomyces hordeovulneris*  
Host: dogs  
Conditions: cutaneous and visceral abscessation, pleuritis, peritonitis, arthritis
- *Actinomyces viscosus*  
Host: dogs  
Conditions: canine actinomycosis

- cutaneous pyogranulomas
- pyothorax and proliferative pyogranulomatous pleural lesions
- disseminated lesions (rare)
- *Actinomyces bovis*  
Host: cattle  
Conditions: bovine actinomycosis (lumpy jaw)
- *Actinomyces viscosus*  
Horses: cutaneous pustules  
Cattle: abortion
- *Actinomyces spp* (unclassified)  
Pigs: pyogranulomatous mastitis  
Horses: poll evil, fistulous withers
- *Actinobaculum suis*  
Pigs: cystitis, pyelonephritis

### Diagnosis

- Clinical specimens: exudates, aspirates and tissue samples from post-mortem
- Direct Gram staining of smear may reveal morphological forms of aetiological agent
- Inoculate blood and MacConkey agars and incubate at 37°C for up to 5 days. Different species have peculiar atmospheric requirement for culture
- Identification criteria
  - *Arcanobacterium pyogenes* produce a characteristic hazy haemolysis along streak lines after 24 hours of aerobic incubation. Pin point colonies are seen after 48 hours. Proteolytic, hydrolyses gelatine
  - *Actinomyces bovis*: adhere to agar media and produces no haemolysis
  - *Actinomyces hordeovulneris*: same as *A. bovis*
  - *Actinomyces viscosus*: produce two colony types
    - Large and smooth: V,Y, and T cell configurations
    - Small and rough: short branching filament

- *Actinobaculum suis*: poor haemolysis on ruminant blood agar. Colonies have a shiny raised centre and a dull edge. It is urease positive

### **SPECIES DIFFERENTIATIONS**

Characteristics	<i>Actinomyces bovis</i>	<i>Actinomyces viscosus</i>	<i>Actinomyces hordeovulneris</i>	<i>Arcanobacterium pyogenes</i>	<i>Actinobaculum suis</i>
Morphology	Filamentous branching, some short forms	Filamentous branching, short forms	Filamentous branching, short forms	Coryneforms	Coryneform
Atmospheric requirement	Anaerobic + CO <sub>2</sub>	10% CO <sub>2</sub>	10% CO <sub>2</sub>	Aerobic	Anaerobic
Haemolysis on sheep blood agar	±	-	±	+	±
Catalase production	-	+	+	-	-
Pitting on Loeffler's serum slope	-	-	-	+	-
Granules in the pus	Sulphur granules	White granules	No granules	No granules	No granule

- Granules in lesion is caused by *A. bovis* contains characteristic clubs. Club colonies are also produced by *Actinobacillus ligniersii* and *Staphylococcus aureus* botryomycosis

## ***RHODOCOCCUS EQUI***

- Gram-positive aerobic bacteria
- Non-motile catalase-positive, oxidase-negative
- Weakly acid fast
- Grows on non-enriched media
- Rod or coccibacillus in shape
- Produces pigments, colonies are pink
- It forms capsule. Produces large, moist, viscid/mucoid colonies

### Diseases

Foals of 1 to 4 months of age: suppurative bronchopneumonia and pulmonary abscessation

Horse: superficial abscessation

Pigs, Cattle: mild cervical lymphadenopathy

Cats: subcutaneous abscesses, mediastinal granulomas

### Diagnosis

- Specimens: tracheal aspirates, pus from lesion
- Inoculate blood and MacConkey agar
- Incubate aerobically at 37 °C for 24 to 48 hours
- No growth on MacConkey
- Does not ferment carbohydrate
- Does not haemolyse on blood agar. It is cAMP test positive. (enhanced haemolysis) with *S. aureus*
- Most strains are urease and H<sub>2</sub>S positive

### **Tutorial Questions**

- 1 Describe the type of colouration produced when *Listeria monocytogenes* colonies are viewed under oblique illumination

- 2 What is the significance of Anton's test in the diagnosis of *Listeria monocytogenes*
- 3 Describe the cold enrichment procedure for the diagnosis of *Listeria monocytogenes*
- 4 What is the aetiological agent of diamond skin disease of pigs
- 5 List two selective media for the isolation of *Corynebacterium spp*
- 6 What staining technique is employed for the demonstration of *Corynebacterial metachromatic granules*

## **PSEUDOMONADACEAE**

- Pathogenic members that infect animals include:
  - Pseudomonas aeruginosa*
  - Burkholderia mallei*
  - Burkholderia pseudomallei*
- Gram negative rods of medium size
- Obligate aerobes
- Oxidase-positive and catalase-positive
- *Pseudomonas species* and *Burkholderia pseudomallei* are motile by polar flagella
- *Burkholderia mallei* is non-motile and require 1% glycerol for enhanced growth
- *P. aeruginosa* produces pigments which diffuse into culture media
- Pigments of *P. aeruginosa* include:
  - Pyocyanin: blue-green
  - Pyoverdin: greenish-yellow
  - Pyorubin: red
  - Pyomelanin: brownish-black

### Diseases

- *P. aeruginosa*: causes opportunistic infection in many species of animals
  - Cattle: mastitis, metritis, pneumonia, calve enteritis, dermatitis
  - Pigs: Ear infection, respiratory tract infection
  - Horses: genital tract infection, pneumonia, eye infection
  - Sheep: mastitis, pneumonia, otitis media, fleece rot/ suppurative dermatitis (predisposing factor: heavy rainfall)
  - Dogs and Cats: pneumonia, ulcerative keratitis, cystitis, otitis externa
  - Minks: haemolytic pneumonia, septicaemia, farmed minks very susceptible
  - Rabbits: pneumonia, septicaemia

Reptiles: necrotic stomatitis, especially in captive reptile (found in oral cavity of snakes)

- *Burkholderia mallei*: glanders (a contagious disease of equidae characterized by the formation of nodules and ulcers in the respiratory tracts or on the skin)
- *Burkholderia pseudomallei*: causes melioidosis-chronic debilitating disease with disseminated abscesses in many organs of the body
- *Pseudomonas fluorescens* and *P. putida*: pathogens of freshwater fish

### Diagnosis

- Sample collection: based on observed clinical signs and lesions. Samples may include pus, respiratory aspirates, ear swab, mastitic milk, discharges, blood (for serology) etc.
- Inoculate blood agar and MacConkey agar plates
- Incubate aerobically for 24 to 48 hours at 37°C
- *B. mallei* grows on media containing 1% glycerol and also on MacConkey agar
- Identification criteria:
  - Colonial morphology
  - Microscopy
  - Biochemical reactions
- Serology
  - Complement fixation test and agglutination technique for *B. mallei* detection
  - Slide agglutination, ELISA, CFT, indirect haemagglutination test used for detection of *B. pseudomallei* serum antibodies
- The mullein test: an efficient field test for screening and confirmation of glanders in animals. Mallein is a glycoprotein extract of *B. mallei*
  - It is injected intradermally just below the lower eyelid
  - A local swelling with mucopurulent ocular discharge is evident after 24 hours in positive cases
- *P. aeruginosa*: produces pigments detectable in media that contains no dye e.g. nutrient agar. It also has a characteristic fruity, grape-like odour

**Comparative features of the Pseudomonadaceae**

Feature	<i>P. aeruginosa</i>	<i>B. mallei</i>	<i>B. pseudomallei</i>
Colonial morphology	Large and flat with serrated edges	White and smooth becoming granular and brown with age	Range from smooth and mucoid to rough and dull becoming yellowish brown with age
Haemolysis on blood agar	+	-	+
Diffusible pigment production	+	-	-
Colony odour	Grape-like	None	Musty
Growth on MacConkey agar	+	+	+
Growth at 42 <sup>o</sup> C	+	-	+
Motility	+	-	+
Oxidase production	+	±	+
Oxidation of carbohydrate:			
Glucose	+	+	+
Lactose	-	-	+
Sucrose	-	-	+

## ENTEROBACTERIACEAE

- Members are Gram-negative rods about 3 µm in length
- Oxidase-negative, catalase-positive
- Ferment glucose and a variety of other sugars
- Non-sporing facultative anaerobes
- Reduce nitrates to nitrites
- Mostly enteric organisms
- Motile members possess peritrichous flagella
- Grow well on MacConkey agar because they tolerate bile salts
- Categorised into two broad groups based on lactose fermentation
  - Lactose fermenters e.g. *E. coli*, *Klebsiella spp*
  - Non-lactose fermenters e.g. *Salmonella spp*, *Proteus spp*
- Major animal pathogens (cause both enteric and systemic diseases)
- Examples:
  - *E. coli*
  - *Salmonella* serotype
  - *Yersinia spp*
    - *Y. pestis*
    - *Y. enterocolitica*
    - *Y. pseudotuberculosis*
    - *Y. intermedia*
    - *Y. kristensenii*
    - *Y. frederiksenii*
    - *Y. ruckerii*: pathogen of fish
- Opportunistic pathogens cause disease outside the GIT
- Major pathogens, cause disease in both enteric and non-enteric locations

*Yersinia species:*

- Yersiniae stain bipolar on primary isolation
- Yersiniae are intracellular organisms localizing in macrophages
- o *Y. pestis:*
  - It is pleomorphic
  - It produces little or no turbidity and small deposit in broth culture
  - Haemin required for aerobic growth on nutrient agar
  - Two forms of colony: smooth and rough
  - Causes plaque: bubonic plaque, (septicemic, pneumonic sylvatic forms).  
Characterized by lymphadenitis
  - Virulence factor F1 or fraction I (capsular/envelope heat-labile protein), V (protein), W (lipoprotein), F (factor antigens)
  - Probably produces toxin
  - Virulence strains kill mice or guinea pigs following intraperitoneal or subcutaneous injection with as low as 10 viable organisms
  - Transmission: Wild rat (through flea) to town rat (through flea) to humans

Diagnosis

- blood sample, materials from lymph nodes
- grow on blood agar and selective media
- Fluorescent antibody test on cerebrospinal fluid and in aspirates

Note:

- Colonies of non-lactose fermenting bacteria are alkaline due to utilization of peptone in medium. They are pale
- Colonies of lactose fermenters are pink due to acid production from lactose
- Somatic (O), flagellar (H), and capsular (K) antigens are used for serological identification and classification of the enterobacteriaceae

### Differentiation and Identification of the Enterobacteriaceae

	<i>E. coli</i>	<i>Salmonella</i> serotype	<i>Yersinia</i> <i>species</i>	<i>Proteus</i> <i>species</i>	<i>Enterobacter</i> serotype	<i>Klebsiella</i> <i>pneumonia</i>
Clinical importance	Major pathogens	Major pathogen	Major pathogen	Opportunistic pathogen	Opportunistic pathogen	Opportunistic pathogen
Cultural characteristics	Some strains haemolytic	-	-	Swarming growth	Mucoid	Mucoid
Motility at 30 <sup>o</sup> C	Motile	Motile	Motile	Motile	Motile	Non-motile
Lactose fermentation	+	-	-	-	+	+
IMV <sub>i</sub> C test						
Indole production	+	-	V	±	-	-
Methyl red test	+	+	+	+	-	-
Voges Proskauer test	-	-	-	V	+	+
Citrate utilization test	-	+	-	V	+	+
H <sub>2</sub> S production in TSI agar	-	+	-	+	-	-
Lysine decarboxylate	+	+	-	-	+	+
Urease production	-	-	+	+	-	+

#### *Yersinia pseudotuberculosis*

- Causes infection in many animals including guinea pigs, mice, rats, rabbits, chicken, turkey, pigeons, and canaries
- Sporadic cases reported in horses, cattle, sheep, goats, pigs and cats
- Produced in necrotic nodules in ileum and caecum as well caseous necrosis of mesenteric lymph nodes and omentum

- Grows on blood agar, MacConkey and Salmonella-shigella agar at 37°C and at room temperature (22°C – 28°C)
- Samples of isolation of organism: liver, spleen, heart blood

#### *Yersinia enterocolitica*

- Grows on blood agar, Salmonella-shigella agar, desoxycholate citrate agar (DCA) and MacConkey agar
- May require enrichment in phosphate buffered solution (pH 7.6) or peptone broth at 4°C for 3 weeks
- Must be differentiated from *Pasteurella*

*Note the following characteristic of Pasteurella: MR negative, Oxidase positive, no growth on MacConkey except Mannheimia haemolytica*

- *Yersinia enterocolitica* grows at 4°C unlike other enteric bacteria
- Pig is a major reservoir
- Isolation requires enrichment then subculture on agar then do identification tests.

#### *Proteus*

- *P. vulgaris*
- *P. mirabilis*
- Pathogenic role doubtful
- May cause diarrhoea in young animals
- Otitis media in dogs
- Often causes infection only when found outside the intestinal tract
- Associated with chronic urinary tract infections

#### Diagnosis

- Produces characteristic smell and swarms on solid media

#### *Klebsiella*

##### *K. pneumoniae*

- Pneumoniae in humans
- Klebsiella and Enterobacter cause neonatal meningitis in children
- Opportunistic infections in animals
- Pneumonia in fowls, metritis in mare and sow
- Mastitis (chronic) in cow
- Complicate air-sac infection and pullorum disease in poultry
- Other species: *K. ozaenae*, *K. rhinoscleromatis*

*Providencia*

*P. stuartii*, *P. rettgeri*, *P. alcalifaciens*

- Involved in urinary tract infection, sepsis, pneumonia and wound infections
- Hospital infection

*Morganella*

*M. morganii*

- Hospital infection
- Implicated in summer diarrhoea in children

**Biochemical differentiation of *Proteus* species**

	<i>Proteus vulgaris</i>	<i>Proteus mirabilis</i>	<i>Providencia rettgeri</i>	<i>Morganella morganii</i>
Maltose fermentation	+	-	-	-
Mannitol fermentation	-	-	Delayed	-
Indole production	+	-	+	+
Gelatin liquefaction	+	+	-	-
H <sub>2</sub> S production	+	+	-	+
Citrate utilization	-	-	+	-

Urease production	+	+	-	+
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### *Salmonella*

Selective media:

- Desoxycholate citrate agar: slightly opaque often with central black spot
- Brilliant-green agar: *S. typhi*, *S. gallirum*, *S. pullorum*, *S. cholerae-suis* and *S. typhi-suis* do not grow on the agar. Colonies are pale-pink usually surrounded by a pink zone. Colonies have a translucent dew-drop appearance
- Wilso and Blair agar: colonies are black
- Salmonella-shigella agar: colonies are pale or colourless
- Hektoen enteric agar: blue-green with black centre
- Motile except *S. galinarium* and *S. pullorum*
- Enrichment media:
  - Selenite F. broth
  - Tetrathionate broth
  - Rappaport broth

### Reactions of Members of Enterobacteriaceae in Triple Sugar Iron (TSI) agar

Species	pH change		H <sub>2</sub> S production
	Slant	Butt	
<i>Salmonella</i> serotype	Red (alkaline)	Yellow (acid)	+
<i>Proteus mirabilis</i>	Red	Yellow	+
<i>P. vulgaris</i>	Yellow	Yellow	+
<i>E. coli</i>	Yellow	Yellow	-
<i>Yersinia enterocolitica</i>	Yellow	Yellow	-
<i>Y. pseudotuberculosis</i>	Red	Yellow	-
<i>Y. pestis</i>	Red	Yellow	-
<i>Enterobacter aerogenes</i>	Yellow	Yellow	-

<i>Klebsiella pneumoniae</i>	Yellow	Yellow	-
<i>Shigella species</i>	Yellow	Red	-

### *Shigella*

- Non-motile
- Non-sporing
- Non-capsulated
- Oxidase-negative, catalase-positive
- *Shigella dysenteriae* type I is catalase negative
  - Species
  - *Sh. dysenteriae* (Tropics): dysentery in human and monkey (shigellosis, colitis)
  - *Sh. flexneri* (Tropics): dysentery in human and monkey (shigellosis, colitis)
  - *Sh. boydii* (Tropics): dysentery in human and monkey (shigellosis, colitis)
  - *Sh. sonnei* (temperate): dysentery in human and monkey (shigellosis, colitis)

### Diagnosis

- Sample: fresh stool
- Small colonies on DCA and MacConkey agar
- *Shigella dysenteriae* type I does not grow on DCA
- No growth on Wilson and Blair medium
- Grow on S-S agar and Hektoen enteric agar producing pale and green colonies respectively
- May be inhibited to a certain extent by selenite F broth

### Biochemical reactions:

Glucose fermentation	Positive (acid only)
Lactose fermentation	Negative
Sucrose fermentation	Negative
Mannitol fermentation	Variable
Indole production	Variable

MR reaction	Positive
Voges-Prostkauser	Negative
Citrate utilization	Negative
H <sub>2</sub> S production	Negative
Urease production	Negative
Motility	Negative

### Biochemical differentiation of *Shigellae*

Test	<i>Sh. dysenteriae</i>	<i>Sh. Flexneri</i>	<i>Sh. boydii</i>	<i>Sh. Sonnei</i>
Glucose	Acid (A)	A/A and G (gas)	Acid	Acid
lactose	-	-	-	Late fermenter
Mannose	-	Acid	Acid	Acid
Sucrose	-	-	-	-
Dulcitol	-	-/A	-	-
Xylose	-	-	-	-
ONPG test	-/+	-	-	+
Indole	Variable	Strain variation	Variable	-

ONPG: Orthonitrophenol (-β-D-galactopyranoside)

### ***ESCHERICHIA COLI***

#### *E. coli* diseases (enteric and extraintestinal)

- Enteric colibacillosis
- Colisepticaemia
- Oedema disease in pigs
- Post-weaning diarrhoea in pigs
- Coliform mastitis
- Urinogenital tract infection

#### Other diagnostic procedure

- Serology/serotyping

- PCR
- Toxin detection
  - Cytotoxicity
  - Loop ligation test
  - Sereny test (invasiveness)
- Animal inoculation

### Salmonella diseases

- Septicaemic salmonellosis
- Enteric salmonellosis
- Fowl typhoids
- Pullorum (bacillary white diarrhoea)
- Ipuman infection
- Abortion in cattle

### Diagnosis

#### Sample from Suspected Animals

- Tissue
- Faeces:
  - Inoculation into enrichment broth e.g. selenite F, rappapoort, Tetrathionate (37°C for 48 hours aerobically)
  - Subculture at 24 and 48 hours onto MacConkey agar, brilliant green and xylose-lysine-deoxycholate
  - Direct inoculation: MacConkey agar, brilliant green and xylose-lysine-deoxycholate (37°C for 24 hours aerobically)
    - Suspicious colonies
    - Inoculation of TSI agar and lysine decarboxylase broth
    - Typical salmonella reactions
    - Serological confirmation with polyvalent antisera
    - Definitive serotyping into specific 'O' and 'H' antisera
    - Biotyping or Phagotyping

*E. coli*

Commensal

Opportunistic

Enteric

Extraintestinal:

Urogenital (uropathogenic), cystitis

Avian

Mastitis

Pyometria (dogs and cats)

Septiceamic (endotoxin): cystitis mainly in bitches

Virulence factors of *E. coli*

CNF: Cytotoxic Necrotizing Factor

ETEC: Adhesins, K88 pigs, and K99 calves and lambs for colonization, heat stable enterotoxins (ST), and heat labile enterotoxins (LT)

Diarrhoea in neonatal piglets, calves, lambs, post-weaning diarrhoea in pigs

EPEC: A/E factor, intimin, haemolysin, destruction of microvilli, shedding of enterocytes, stunting of villi malabsorption, diarrhoea in piglets, lambs, pigs

VTEC: VT1, VT2, VT2e, damage to vasculature in intestine and other locations, oedema disease in pigs, haemolytic colitis in calves, post-weaning diarrhoea in pigs, haemolytic uraemic syndrome (HUS) and haemorrhagic colitis (HC) in humans

Necrotoxicogenic: CNF1 and CNF2 (Cytotoxic Necrotizing Factor). Damage to enterocytes and blood vessels, HC in cattle, enteritis in piglets and calves, diarrhoea in rabbits, dysentery in horses

### ***BACILLUS SPECIES***

- *Bacillus species* are large Gram-positive rods about 10.0 µm in length
- They produce endospores
- They appear singly, in pairs or in long chains
- They are aerobic or facultative anaerobes
- *Bacillus species* are catalase-positive and oxidase negative
- They are motile except *B. anthracis* and *B. mycoides*
- Most species are saprophytes but often contaminate clinical specimen and laboratory media
- *B. species* can tolerate extreme adverse conditions such as high temperature and desiccation because of their endospores
- *B. anthracis* produces capsule

#### Diseases

##### *B. anthracis*

Cattle and sheep: total peracute or acute septicaemic anthrax

Pigs: subacute anthrax with oedematous swelling in pharyngeal region, intestinal form with higher mortality is less common

Horses: subacute anthrax with localised oedema, septicaemia with enteritis and colic

Human: skin, pulmonary and intestinal forms of anthrax

##### *B. cereus*

Cattle: mastitis

Human: food poisoning, eye infection

##### *B. licheniformis*

Cattle, sheep: sporadic abortion

#### Diagnosis:

- Ability to produce catalase and grow aerobically distinguish *B. species* from *Clostridium spp*
- *Bacillus species* are differentiated based on colonial characteristics, biochemical test and genetic composition
- Colonial characteristics:
  - *B. anthrax* colonies are up to 5mm in diameter, flat, dry, greyish and with a 'ground-glass' appearance after 48 hours incubation. At low magnification, curled outgrowth from the edge of the colony impart a characteristic 'medusa head' appearance. Isolates are rarely haemolytic. When present, haemolysis is weak
  - *B. cereus*: colonies are similar to those of *B. anthracis* but slightly larger with a greenish tinge. The majority of strains produce a wide zone of complete haemolysis around the colonies
  - *B. licheniformis*: colonies are dull, rough, wrinkled and strongly adherent to the agar. Characteristic hair-like outgrowth are produced from streaks of the organisms on agar media

**Distinguishing features of *B. anthracis* and *B. cereus***

Feature	<i>B. anthracis</i>	<i>B. cereus</i>
Motility	Non-motile	Motile
Appearance on sheep blood agar	Non-haemolytic	Haemolytic
Susceptibility to penicillin	Susceptible	Resistant
Lecithinase activity on egg yolk agar	Weak and slow	Strong and rapid
Effect of gamma phage	Lysis	Lysis rare
Pathogenicity for animals	Death in 24 -48 hours	No effect

### Diagnosis of Anthrax:

- History of sudden death
- Pathology: carcass is bloated, putrefies rapidly and no rigor mortis
- Collect peripheral blood and make smear
- Stains smear with polychrome methylene blue
- *B. anthracis* appears as blue-staining rods with square-end surrounded by pink capsules
- Culture on blood and MacConkey agar
- Incubate aerobically at 37°C for 24 to 48 hours
- Study colony morphology
- No growth on MacConkey agar
- Study microscopic appearance
- Do biochemical test
- Conduct pathogenicity test
- Do Ascoli test:
  - o A thermoprecipitation test
  - o Detects *B. anthracis* antigen
  - o A ring precipitation or gel diffusion test with *B. anthracis* antiserum
- Other tests: agar gel immunodiffusion, CFT, ELISA, IFT and PCR

### ***CLOSTRIDIUM SPECIES***

- Large gram-positive rods
  - Produces endospores. *C. perfringens* rarely produce spores
  - Anaerobic
  - Catalase and oxidase negative
  - Motile except *C. perfringens*
  - Require enriched media for growth
  - Size, shape and location of endospores used for species differentiation
  - They are toxigenic. They are non-capsulated except *C. perfringens*
- C. perfringens*: large wide rods. Rarely form endospores in-vitro

*C. tetani*: thin rods. Characteristically produce terminal endospores (drumstick appearance)

*C. chauvoei*: medium-size rods. Produce lemon shaped endospores

### Diseases:

Categorised into three major groups based on toxin activity

- Neurotoxic clostridium: *C. tetani*, *C. botulinum*
- Histotoxic clostridia: localized lesion in liver and muscle: *C. chauvoei*, *C. septicum*, *C. novyi type A*, *C. perfringens type A*, *C. sordelli*, *C. haemolyticum*, *C. novyi type B*
- Enterotoxigenic clostridia: *C. perfringens type A-E*
- Less important groups
  - o *C. piliforme*: spore-forming, filamentous gram-negative intracellular pathogens (atypical member of the clostridia). Has not been cultured artificially on media
    - Grows only in tissue culture and fertile egg
    - Causes Tyzzer's disease (a severe disease causing hepatic necrosis) in laboratory animals foals rarely in calves, dogs and cats
  - o *C. difficile*: chronic diarrhoea in dogs and haemorrhagic enterocolitis in newborn foals
  - o *C. spiroforme*: enteritis in rabbits
  - o *C. colinum*: enteritis in birds
- Neurotoxic clostridia:
  - i. *C. tetani*: locked jaw/tetanus
    - Infect wounds
    - Terminal endospores
    - Toxin produced in wounds
    - Toxin production in regulated by genes encoded in plasmids
    - One antigenic type of toxin (tetanus plamin)
    - Toxin causes synaptic spasms
    - Prevented by toxoid
    - Treated by antitoxin

- ii. *C. botulinum*: botulism
  - subterminal endospores
  - preformed toxin in canned foods, carcasses, decaying vegetation etc.
  - toxin production regulated by genomes
  - eight antigenically distinct toxins (A-G)
  - toxins inhibit neuromuscular transmission
  - produces flaccid paralysis
  - most potent biological toxin known
  - prevented by toxoids, treated by antitoxin
- histotoxic clostridia: they produce toxins ( $\alpha, \beta, \gamma, \delta$  toxins)
  - *C. chauvoei* ( $\alpha, \beta, \gamma, \delta$ ): blackleg in cattle and sheep.
  - *C. septicum* ( $\alpha, \beta, \gamma, \delta$ ): malignant oedema in cattle, pig and sheep. Braxy (abomastitis) in sheep and occasionally calves
  - *C. noryi* type A ( $\alpha$ ): big head in young rams, wound infection
  - *C. sordell* ( $\alpha, \beta$ ): myositis in cattle, sheep, horses, abomastitis in lambs
  - *C. novyi* type B ( $\alpha, \beta$ ), infectious necrotic hepatitis (black disease) in sheep and occasionally in cattle
  - *C. haemolyticum* ( $\beta$ ): bacillary haemoglobinuria in cattle and occasionally in sheep
  - *C. perfringens* type A ( $\alpha$ ): necrotic enteritis in chicken, necrotizing enterocolitis in pigs, gas gangrene.
- Enterotoxaemia clostridia: toxins ( $\alpha, \beta, \epsilon, \iota$ ) *C. perfringens* type A – E
  - Type A ( $\alpha$  toxin): necrotic enteritis in chicken, necrotizing enterocolitis in pigs, canine haemorrhagic gastroenteritis
  - Type B ( $\alpha, \beta$  (major),  $\epsilon, \iota$ ): lamb dysentery; haemorrhagic enteritis in calves and foals
  - Type C ( $\alpha, \beta$  (major)): struck in adult sheep, necrotic enteritis in chickens, haemorrhagic enteritis in neonatal piglets, sudden death in goats and feedlot cattle

- Type D ( $\alpha$ ,  $\epsilon$  (major)): pulpy kidney in sheep, enterotoxaemia in calves, adult goats and kids
- Type E ( $\alpha$  and  $\iota$ (major)): haemorrhagic infection in calves, enteritis in rabbits

### Diagnosis

- Clostridia are fastidious and anaerobic
- Samples are collected from live or recently dead animals
- Tissues or exudates for culture should be placed in anaerobic transport media
- Samples should be cultured promptly
- Ideal medium is blood agar enriched with yeast extract, vitamin K and haemin
- Robertson cooked medium is used for anaerobic enrichment
- Media should be freshly prepared or pre-reduced to ensure absence of oxygen
- Test for toxin production in laboratory animals. Toxin neutralisation by antitoxin.
- Cultured plates are incubated in anaerobic jar containing hydrogen supplemented with 5 to 10% carbon dioxide
- Identification and differentiation among *C. species* are based on colonial morphology, biochemical tests, toxin neutralization methods and gas-liquid chromatography for profiling organic acids
- Fluorescent antibody techniques, immunoassay such as ELISA and molecular technique like PCR are of diagnostic importance

### Special Features

*C. tetani* produces filmy growth on blood agar with narrow zone of haemolysis. Prevent swarming by using 4% agar (stiff) or sodium azide

*C. perfringens*: produces double zone of haemolysis on blood agar (narrow zone of incomplete haemolysis and wide zone of partial haemolysis). Produces marked opalescence on egg yolk medium because of the lecithinase action of alpha toxin (Nagler reaction).

CAMP test positive with *Streptococcus agalactiae*

*C. novyi type A*: give a characteristic 'pearly layer' on egg yolk medium due to the lipase it produces. It is also Nagler's reaction

Principle of Nagler's reaction: lecithinase action on lecithin in egg yolk leading to the opacity due to insoluble fatty acid accumulation

### **Tutorial questions**

1. list the steps involved in bacteria infection
2. what is virulence
3. how do bacteria obtain iron from the host
4. List three bacteria that can survive in macrophages
5. what is the significance of coagulase production by bacteria
6. list five differences between exotoxins and endotoxins
7. what is the causative agent in tick pyaemia in lamb
8. what selective medium will you use for the isolation of staphylococcus
9. list three differences between staphylococci and streptococci
10. what is lancefield grouping

**VETERINARY BACTERIOLOGY (VPM 401) LECTURE NOTES  
DEPT. OF VETERINARY MICROBIOLOGY AND PARASITOLOGY  
COLLEGE OF VETERINARY MEDICINE  
UNIVERSITY OF AGRICULTURE ABEOKUTA, OGUN STATE,  
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**NEISSERIAE AND BRANHAMELLA**

**Neisseriae**

They are Gram-negative cocci, usually occurring in pairs. Some species of the genus are normal inhabitants of the human and animal respiratory tracts, and are extracellular. *N. gonorrhoeae* and *N. meningitidis* are human pathogens, which are intracellular.

**General Characteristics**

The typical pathogenic *Neisseria* organism is a gram- negative diplococcus, approximately 0.8µm in diameter. Individual cocci are Kidney-shaped, with the flat or concave sides adjacent. *N.gonorrhoeae* and *N.meningitidis* autolyze quickly, particularly in alkaline environment.

### **Media and Growth**

The organisms grow well on enriched media, the pathogenic ones are normally cultured on selective media. *N. gonorrhoeae* and *N. meningitides* grow on best media containing complex organic substances such as blood or animal proteins in an atmosphere of 10 percent CO<sub>2</sub>. They prefer solid medium to liquid but synthetic media have been developed.

Liquid media have been devised for culture of the organisms in large quantities. They grow well on chocolate agar but a selective medium popularly used in the laboratories is the Thayer-Martins medium. The medium contains sodium colistimethate and vancomycin to inhibit the growth of bacterial contaminants. Nystatin is also added to prevent the growth of fungal contaminants.

### **Species**

The other species of *Neisseria* include *N. flavescens*, *N. flava*, *N. sicca*, *N. pharyngis*, *N. canis*, *N. ovis*, and *N. lactamica*. They are generally regarded as nonpathogenic. The nonpathogenic species can grow at the low temperature of 22<sup>0</sup>C while the pathogenic species have optimum temperature of 35<sup>0</sup>C-36<sup>0</sup>C, minimum and maximum temperatures of 30<sup>0</sup>c and 38<sup>0</sup>c respectively.

### **Biochemical reactions**

The pathogenic ones ferment very limited carbohydrates producing acid but not gas from them. For biochemical tests, serum slope sugars, with 5 percent human, rabbit or guinea pig serum plus

1 percent carbohydrate, are preferred. Horse serum is not used because it may contain maltase, which splits maltose, thereby giving false positive result. The non pathogenic species are biochemically active. *Neisseria* species are oxidase and catalase positive, indole and methyl red (MR) negative.

### **CAMPYLOBACTER AND HELICOBACTER**

**Campylobacter:** Mcfadyean and Stockman in 1913 first isolated the organisms of the genus *Campylobacter*, but they were classified as vibrios on account of their curved shape and rapid motility. Because they are associated with infectious infertility and abortion in cattle and sheep, they were named *Vibrio fetus*. By 1963, Sebald and Veron showed that the campylobacters were sufficiently different to warrant separation into a new genus, hence the name *Campylobacter* (Greek; meaning curved rod). Apart from being nonsaccharolytic and microaerophilic, the organisms have G + C content of 30-35 mol percent while for vibrios it is 48 mol percent.

Members of the genus are Gram-negative, microaerophilic curved or spiral rods, with flagella at both ends of the cell which are responsible for the unique cork screw motility of the organisms. In direct smears from clinical materials, they appear S-shaped or may have a “seagull” appearance. Organisms from cultures are longer and more variable. They may form spherical or coccoid bodies.

Colony forms vary but basic types on blood agar are shiny, pale, grey, semi-translucent, flattened and nonhaemolytic. The colonies have “running” appearance. Growth on solid media is generally delicate, colonies may coalesce and sometimes appear as a slight frosting on solid media. Biochemically they are relatively inactive. They do not ferment any carbohydrates but they are oxidase positive while some produce catalase.

**Media:** Media commonly used include blood agar, brucella medium and brain heart infusion broth and agar, the later is supplemented with 5 per cent blood.

Selective media are used for primary isolation of campylobacters in order to suppress contaminants. Basic selective media are based on Skirrow's or Butzler's medium. They are usually supplemented with antibacterial agents, such as, vancomycin (10µg/ml), polymycin B (2.5 i.u/ml), timethoprim (5 µg/ml), novobiocin (5µg/ml) and cephalothin (15 µg/ml). These media are commercially available.

Generally, camylobacters require reduced oxygen tension (microaerophilic) for growth. It is often reduced to 1/3 of normal and a common gas mixture is 10 per cent CO<sub>2</sub>, 5 percent O<sub>2</sub> and 85 per cent nitrogen. Incubation is at 37<sup>0</sup> – 42<sup>0</sup>C and incubated cultures are examined daily up to 7 days. The thermophilic campylobacters grow at 37<sup>0</sup>C and 42<sup>0</sup>C and not as 25<sup>0</sup>C, whereas the non-thermophilic grow at 37<sup>0</sup>C and 25<sup>0</sup>C but fail to fail at 42<sup>0</sup>C. *C. jejuni* and *C. coli* will grow at 42<sup>0</sup>C and at 37<sup>0</sup>C.

**Camylobacteriosis:** Refers to disease conditions due to *Campylobacter* species.

**Humans:** The important species associated with diseases in humans are *C. jejuni* and *C. coli*. The campylobacters have been associated for many years with healthy and diseased domestic animals and poultry. Because of this, it has been suggested that animals or animal products are the most frequent sources of *C. jejuni* and *C. coli* infections in humans. Common sources of *C. jejuni* infections in humans are often associated with fresh poultry, minced meat and unpasteurized milk. Unpasteurized milk, containing about 400 viable cells of *C. jejuni*, when consumed, can cause enteritis.

**Resistance:** The organism is sensitive to acid pH. It is rapidly killed by HCl at pH 2.3, hence the gastric acid is an effective barrier against infection. It can survive for 2-5 weeks in bovine milk or water kept at 4°C. The infective dose varies from 500 to 10,000 organisms in volunteers. The variation may be associated with various factors in the organism and the host.

**Virulence Factors:** Factors associated with virulence and infections include:

- (a) Motility and chemotaxis which are associated with the flagella. Chemotactic stimuli direct motility which enhances the effectiveness of mucosal colonization.
- (b) **Adhesion:** Fimbriae have not been demonstrated in *C. jejuni* and *C. coli* but at least *C. jejuni* is capable of adhesion *in vitro* using HeLa or INT407 cells. The adhesion may be associated with other structures such as outer membrane proteins (OMPs) and lipopolysaccharide (LPS).
- (c) **Enterotoxin:** It has been demonstrated in *C. jejuni*. It is heat-labile. It induces secretory diarrhea by stimulating adenylate cyclase activity in the intestinal mucosa thereby affecting the normal ion transport in the enterocytes. There is some evidence that *C. jejuni* and *C. coli* secrete a cytotoxin which is toxic for mammalian cells, for example, bovine kidney and HeLa cells.
- (d) **Invasiveness:** The organism penetrates intestinal mucosa and proliferates in the lamina propria and mesenteric lymph nodes. This results in low grade damage of the affected tissues. The virulence factors may not be manifested by all strains of *C. jejuni* and this may explain variation in symptoms of Campylobacter enteritis.

*C.jejuni* infection is most commonly gastrointestinal. A few uncommon cases of extraintestinal infections have been reported. They include meningitis, cholecystitis and reactive arthritis. Infections in humans were not reported until the early 1970s. pioneer workers in human infections are Skirrow or Britain and Butzler of Belgium, *Campylobacter* enteritis has now been documented in many countries.

**Laboratory diagnosis:** Bacteriological diagnosis is based on isolation and identification of *C. jejuni* and *C. coli*. *C. jejuni* is the more important pathogen and frequently associated with *Campylobacter* enteritis.

Direct field microscopy of stool specimens may be carried out for presumptive identification. Care must be taken not to misdiagnose the organism for *V.cholerae*. *Campylobacter* sp. shows corkscrew motility while *V.cholrae* exhibits darting motility. The later is coma-shaped while the former is spiral or s-shpaed. Immunofluorescent technique can be used to detect *C. jejuni* in various specimens. Other tests which are promising are ELISA and bacteriophage typing using c-phages specific for *C. jejuni*.

**Virulence factors:** They are associated with the cell wall LPS which mediates resistance to the bactericidal activity of bovine serum. The other factor is the antigenic variation manifested in the surface components of the organism. The variation occurs in *vivo* enabling the organism to persist in the uterus and vagina.

Infection is acquired during coitus or by artificial insemination procedures. Bull to bull transmission may take place during mounting when many bulls are enclosed together. Bovine

venereal campylobacteriosis is a chronic infection of the female genital tract, characterized by mild endometritis and transient infertility. The infection is confined primarily to the surfaces of the mucous membrane. Soon after infection the organism can be found in the vagina, the cervix, uterus and oviduct of susceptible cows. The infection is transient in the uterus but becomes established in the cervix and vagina. Abortion may be due to bacterial inflammatory placentitis or allergic response to endotoxin of the organism. The endotoxin has been shown to be abortifacient in pregnant cows.

The earliest antibodies to appear are IgM followed by IgG and IgA. IgG predominates in the uterine secretion of convalescing animals, and IgA is found in the cervico-vaginal secretions. IgA helps to immobilize the organism thereby limiting its entry to the uterus while IgG plays a role in opsonization during phagocytosis.

The organism persists in the vagina for up to 2 years. The persistence may be associated with antigenic variation resulting from phase conversion. Asymptomatic vaginal carrier may arise in animals which regained their fertility but continue to harbour the organism in the vagina or in convalescent animals which have become susceptible to reinfection due to decline or loss of immunity.

### **Laboratory diagnosis**

(a) Bacteriological diagnosis in the bull is carried out by culture of the perputial materials.

From the cow, materials are obtained from the vagina and cervix by aspiration. In the case of abortion, specimens are obtained from the placenta, cotyledon and the aborted foetus including the stomach content. Cultures are made on selective media. Suspected colonies may be identified by the fluorescent antibody technique or biochemically.

(b) Serological test

- (i) Vaginal mucus agglutination (VMA) is useful as a herd test but of little value in identifying individual infected animals.
- (ii) Indirect haemagglutination (IHA) using tanned sheep red blood cells, sensitized with phenol-extracted antigen. False positive results may occur in about 1 percent of mucus samples from non-infected cattle.
- (iii) Immunofluorescent technique. It is a useful rapid screening method as an adjunct to cultural examination. It can not distinguish the two subspecies though it is specific for *C. fetus*.

*Campylobacter fetus ss fetus*. The organism has been isolated from gall bladder, intestinal tract and occasionally from the genital tract of healthy animals. The important infections in animals are abortion and enteritis.

### **VIBRIO, AREOMONAS AND PLESIOMONAS**

**Vibrio:** The organism of the genus are Gram-negative, nonsporing curved rods, with single polar flagella. They are oxidase and catalase positive. Generally they ferment glucose with acid production only. They are aerobic and facultative anaerobic, some are proteolytic and liquefy gelatine. The type species is *V. cholerae* isolated by Robert Koch in Egypt in 1883. This group of bacteria and other related organisms may be encouraged in bacteriology laboratory and in some cases false identification may be made.

**Vibrio cholerae:** It is a Gram-negative curved rod with single polar flagella. It is aerobic and facultative anaerobic. It ferments glucose with acid production only and grows well in alkaline pH while the growth is decreased or there may be no growth at all in acid pH. The optimum temperature of growth is 37°C with a range of 15°C to 42°C.

**Growth Media:** *V. cholerae* grows well on ordinary media such as nutrient agar, blood agar or nutrient broth. Selective and enrichment media are also used for the isolation of the organism when contaminated with other enteric pathogens. Alkaline peptone water (pH 8.4) is a good enrichment medium, particularly in field outbreaks. Selective media include, thiosulphate-citrate-bile salt sucrose (TCBS) agar and taurocholate tellurite gelatine agar (TTGA). The two media are popularly used in cholera laboratories.

**Biotypes:** Two biotypes have been identified. They are EI Tor and classic biotypes.

**Antigenic groups:** By the use of the O antigen, three serotypes are known. They are (a) Inaba with antigenic components A and C, (b) Ogawa with components A and B and (C) Hikojima with components A, B and C. The first two are more common. Serotype Ogawa can change to serotype Inaba but the reverse does not occur.

**Resistance:** The resistance is low. It is killed by heat at 56<sup>0</sup>C in 15 minutes, drying and 0.5 percent phenol in a few minutes. It can survive in clean tap water up to 30 days, but chlorinated water, for example “Milton” kills it easily. It fails to grow in acidic medium, for example, gastric fluid. The organism can multiply in foods.

**Other Vibrios**

*V.parahaemolyticus*. it is halophilic marine vibrio, which was first recognize as a cause of food poisoning in Japan in the early 1950s. It shares some characteristics with *V.cholerae* but it tolerates a high concentration of salt in contrast to *V. cholerae*.

It causes enteritis in humans and two types have been described. (1) Water diarrhea with abdominal cramp, nausea, vomiting and fever, and (2) Dysentary-like infection with a shorter incubation period, (2.5 hours or more) than the former (15 hours). In both cases the illness is usually self-limiting. The infection has been reported in many parts of the world. Wound infection with *V.parahaemolyticus* has been reported from Australia, Canada and the USA in persons with wounds exposed to sea-water.

The enteritis due to the organism appears to be transmitted exclusively by food, particularly seafood. The infection is most common during the warmer months. This may reflect both enhanced opportunity for *V.parahaemolyticus* to multiply in unrefrigerated foods and increased prevalence of the organisms in the environment during the warmer months. The organisms multiply very fast (generation time of 9 minutes) under ideal conditions in food and quickly reaches the rather large infectious dose (ID<sub>50</sub>) of  $10^5 - 10^7$  for volunteers. Growth is inhibited at temperatures below 15<sup>0</sup>C and above 65<sup>0</sup>C.

Almost all the isolates from patients with diarrhea and 1 percent of isolates from sea foods and seawater are Kanagawa positive on Wagatsuma agar. However, it is not certain that all Kanagawa-positive strains are pathogenic. The Kanagawa reaction is caused by a heat-stable haemolysin with a molecular weight of about 42,000 daltons. Kanagawa positivity is associated with penetration of the intestinal epithelium of infant rabbits, rapid cytotoxicity in HeLa cell cultures, rapid adhesion to HeLa and human foetal intestinal cells. It is also associated with the production of heat-labile factor that produces a cholera-like reaction in CHO cells.

Other vibrios which have been associated with human infections include: *V. vulnificus*, *V. fluvialis* and *V. mimicus*, *V. anguillarum* and *V. ordalii*.

***V. metchnikovii***: It was originally isolated from the blood and gut contents of chickens dying of fowl cholera-like disease. It grows rapidly in peptone water and grows well on DCA. It is aerobic and facultative anaerobic. Temperature range of growth is 30<sup>0</sup>-40<sup>0</sup>C.

## BRUCELLA

Sir Bruce in 1886 isolated an organism which caused Maltese fever from the liver of a patient who died of the disease, hence the generic name, *Brucella*. The organism was later isolated from goat's milk and consequently name *Brucella melitensis*. Bang isolated similar organism from aborting cows in 1897, known as *B. abortus*. Carmichael and Kenny in 1968 isolated *B. canis* from cases of abortion in the Beagles.

The organisms belonging to the genus are Gram-negative cocco-bacilli, which are nonsporing and nonmotile. They are strict aerobes but some strains require 5-10 percent CO<sub>2</sub> for growth. Although they utilize various carbohydrates, the amounts of acid produced are too small to be of practical use for identifying individual species. *Brucella* species are obligate intracellular organisms.

Media of growth include liver infusion agar, blood agar, chocolate agar, glycerol glucose agar and serum dextrose agar. The latter consists of 5 percent serum and 1 percent dextrose. It is popularly used in the isolation of the organisms.

Antibacterial and antifungal agents may be added to a medium for primary culture. The agents include bacitracin, actidione, fungizone, polymyxin B, cycloheximide and vancomycin. They may be added in varying combinations to suppress the growth of contaminants.

CO<sub>2</sub> requirement.- *B.abortus* and *B. ovis* require optimum 10 percent CO<sub>2</sub> for growth. Although *B. melitensis* and *B. suis* do not require CO<sub>2</sub> for growth, their growths, however, improve with the addition of CO<sub>2</sub>.

Maximum temperature of growth is 37<sup>0</sup>C and growth occurs in 2-4 days. The colonies are small and transparent to translucent. They are catalase positive and some species reduce nitrates to nitrites.

Both *B. abortus* and *B. suis* will agglutinate monospecific antiserum to *B. abortus* while *B. melitensis* agglutinates only its monospecific homologous antiserum.

The resistance of brucellae is low. They are killed by heating at 60<sup>0</sup>C for 10 minutes, pasteurization, acid pH, disinfectants and direct sunlight. They survive for long at low temperatures and can be preserved at -40<sup>0</sup>C.

**Antigens:** Smooth strains of *B. abortus*, *B. suis* and *B. melitensis* each possess two antigens designated A and M. However, there is quantitative difference between these antigens in *B. abortus* and *B. suis* on one hand and *B. melitensis* on the other. The A and M antigens of *B. abortus* occur in a ratio of 20:1 and it is a slightly narrower proportion in *B. suis*, whereas in *B. melitensis* the proportion is 1:20. It is therefore possible to absorb A from *B. melitensis* and M from *B. abortus* by agglutination resulting in monospecific antisera. Crossreactions with other Gram-negative bacteria have been reported. The list of bacteria includes *E. coli* 0116 and 0157, *F.tularensis*, *Salmonella* group N. *Y. enterocolita* 09 and *V. cholerae* 01.

**Bacteriophage typing:** The bacteriophage, Tbilisi (Tb) is specific for smooth strains of *B. abortus* in routine test dilution (RTD). At 10,000 RTD, the phage will lyse *B. suis* but not *B. melitensis*. The phage is stable at 4<sup>0</sup>C for a long period.

**Virulence factors:** The virulence of *Brucella* species is associated with their intracellular location within the reticuloendothelial systems.

**Brucellosis:** It is a disease caused by *Brucella* species in humans and animals. The disease is primarily a disease of animals. Any human infection is an indication of animal infection since the disease is only transmitted from animals to humans. It is an occupation hazard for dairy workers, veterinarians, butchers, animal attendants and farmers.

Infection with *Brucella* organisms is acquired by ingestion, for example, unpasteurized infected milk or through penetration of the mucous membrane of the conjunctiva, the oropharynx and upper respiratory tract by the organism. Cream is a good vehicle of the organism but butter and cheese are not usually associated.

## **Animals**

**Cattle:** *B. abortus* infection occurs in most parts of the world, although some countries particularly in Europe have controlled or eradicated it in their cattle populations.

Infection in a herd often occurs from the introduction of infected cattle. The disease subsequently spreads within the herd due to contamination of the environment from aborted fetuses, genital discharges and placentae. Infection may be transmitted to calves through infected colostrums or milk. The organism may be introduced through other routes to cattle; ingestion, via vaginal

during coitus, conjunctiva, possibly by inhalation and artificially inseminated contaminated semen.

Non-pregnant cows infected with *B. abortus* usually develop infection of the udder and supramammary lymph glands. *Brucellae* have a predilection for the gravid uterus in pregnant animals and hence placentitis resulting in abortion in susceptible animals. Premature births of weak calves usually occur in herds where the disease is endemic. Erythritol enhances the growth of brucellae, the placenta and testicular tissues of cattle, sheep, goats and pigs are rich in erythritol, a saccharide alcohol. This enhances the growth of the organism in the genital tract particularly the placenta in a pregnant animal. The placenta of human does not contain erythritol and this may explain why *Brucella* species do not cause abortion in pregnant women.

In an acute infection, particularly in a susceptible clean herd, “stormy abortion” often occurs with about 50 percent of infected animals aborting. There is a decline in subsequent years. Bulls are commonly infected in the testicles, seminal vesicles and epididymis. This may result in the contamination of semen with *Brucella* organism. Cattle are susceptible to *B. melitensis* and *B. suis* but abortion is not frequent. The organisms, however, establish in the udder and hence their public health importance. *B. abortus* may localize in hygromas of the joints. These cases are seen in Nigeria and the hygroma fluid is rich in *B. abortus*.

**Goats:** The most important *Brucella* species of goats is *B. melitensis*. It causes abortion in goats. It has been reported in goat producing countries, for example, Mediterranean countries, India, Nigeria, Kenya and Tanzania. The organism is excreted in milk for several weeks or months. Goats are susceptible to *B. abortus* infection but it appears not to cause abortion in goats. *B. abortus* has been isolated from goats in Nigeria.

**Sheep:** *B. melitensis* causes brucellosis in sheep and all ages of sheep are susceptible to infection. The organisms usually settle in the genital organs including the mammary glands and the associated lymph nodes, after a transient bacteraemia. Diseased animals spread the infection through aborted fetuses, milk, vaginal discharges and urine.

*B. ovis* has been isolated from rams in New Zealand, Australia and Czechoslovakia. It causes epididymitis in rams. The tails of the epididymis and, to a less extent, the interstitial tissues are the commonest sites for primary infection. Bacteraemia may develop and the organisms are found in the kidney with intermittent excretion in the urine. The semen of the affected animals may also be infected.

**Pigs:** *B. suis* is the normal species causing brucellosis in pigs. *B. abortus* may be isolated from pigs but it is not an important cause of disease in the animal. Swine brucellosis occurs in many countries where pigs are reared and all ages of pigs are susceptible. The organisms are found in the mammary glands as well as in the bladder, spleen and joints. Like *B. abortus*, the gravid uterus is the predilection site for the organism, resulting in abortion in many cases. Hares are susceptible to *B. suis* and they may therefore be important in the epidemiology of swine brucellosis.

**Horses:** *Brucella* organism may be associated with fistulous withers, poll evil and joint infections in horses. *B. abortus* is the usual pathogen isolated with or without any other bacteria.

**Dogs:** *B. canis* specific for dogs. It causes abortion in bitches, particularly among beagles breed, and orchitis in the males.

*B. neotomae* and *B. rangiferitarandi* have been isolated from rodents and snakes respectively. Their roles in the animals are not known.

**Laboratory diagnosis:** Diagnosis of brucellosis in animals is based on microscopy, culture and serology.

(a) **Microscopy:** The foetal stomach content is examined for *B. abortus* in the case of bovine by staining preferably by the modified Ziehl-Neelsen method. The same method is applicable to *B. melitensis* or any other *Brucella* species. *Brucella* organisms stain pink and are coccobacillary. They are usually present in large numbers. In the absence of the foetus, smears are made from the cotyledon or placental materials. In these cases, there may be other bacteria present but *Brucella* organisms normally stain readily by the modified acid-fast staining technique.

(b) **Culture:** the content of the foetal stomach, the placental materials or ground cotyledon is seeded on serum dextrose agar plates or on any other media, with or without antibacterial and antifungal agents. For primary cultures the antibacterial and antifungal agents should be incorporated into the chosen media. The plates are incubated in 5-10 percent CO<sub>2</sub>- enriched atmosphere at 37<sup>0</sup>C for 4 days. If there is no growth after 4 days, the plates are incubated further for another 4 days. Slide agglutination with monospecific serum is then carried out for the identification of the *Brucella* species.

If materials available are contaminated and selective media are not available, such materials may be inoculated peritoneally into guinea pigs. Pure cultures are obtained from the spleens when

cultured on non-selective medium like blood agar. This also has the added advantage that small numbers of organisms can be isolated using guinea pig. Infected guinea pig is necropsied when its serum SAT is 1:20 – 1:40. In male guinea pig, orchitis or Strauss phenomenon occurs when the inoculated material contains *B. abortus*.

**(c) Serology**

(1) Rose Bengal Plate Test (RBPT) is very useful in the field. *Brucella* organisms are stained with rose Bengal stain at pH 3.5. The stained antigen may be obtained commercially or from Reference Laboratories.

A drop of the serum is mixed with a drop of the stained antigen. The mixture is rotated for 2-3 minutes and agglutination occurs within 4 minutes in positive cases. The serum may be diluted up to 1:8 and spot agglutination carried out with each dilution. The test is specific, sensitive and very useful in survey works. The positive sera are transported to the laboratory for further quantitative serological tests.

(2) Serum agglutination test (SAT): It is a reliable test in bovine, caprine and ovine brucellosis. It is less reliable in swine brucellosis diagnosis. The RBPT is more reliable for swine brucellosis test. The SAT is sufficiently standardized regardless of the modification in the test in some countries, that the results can be reported in international units (i.u.). Standard serum can be obtained from the International Reference Centre for Brucellosis, Weybridge, England.

Two –fold dilutions of sera starting with 1:10 dilution are made in 0.5 percent phenol-saline and equal amount of the standard antigen added so that the final volume is 1ml. In addition five tubes containing antigen and saline are prepared to give “changes” of 0, 25,

50, 70 and 100 percent. The tubes are then incubated at 37<sup>0</sup>C in a water bath preferably with a stirrer to provide conventional current to ensure uniform temperature. The incubation is for 18-20 hours or overnight. The tubes are then removed and left at room temperature for 1 hour and then read using a box with lighting, directly at the bottom of the tubes. The “clearings” as well as the titres are read and scored, for example, 1/20<sup>1+</sup>, 1/20<sup>2+</sup>, 1/20<sup>3+</sup> and 1/20<sup>4+</sup>. Both positive (standard serum) and negative controls are included in the test. The titres can be converted to international units. Generally 100 1.u.(1/40<sup>1+4+</sup>) is considered positive for bovine. When *B. abortus* antigen is used in caprine or ovine brucellosis for the SAT, 50 1. u. or above is considered positive .

- (3) Milk ring test (MRT). It is generally carried out on individual animals or on bulk milk sample. For MRT, 1ml of the milk in a 1ml – tube is mixed with a drop of stained suspension of the organism and shaken. The mixture is incubated in a water bath at 37<sup>0</sup>C for 30 minutes or 1 hour. A positive reaction occurs when a blue ring is formed at the top due to antigen – antibody reaction. The cream is white or colourless when the test is negative. False positive results may occur in mastitis cases, particularly in goats. If the milk contains little cream, sterile cream is added to the milk to aid diagnosis.
- (4) Complement fixation test (CFT) and antiglobulin (Coomb’s) test have been used as additional tests. In doubtful SAT cases, CFT is very useful. Coomb’s test is seldom used in livestock animals.
- (5) Whey agglutination test. It is carried out to detect animals which excrete *B. abortus*. Animal must not have been vaccinated at least 3 months prior to the test. The higher the titre the more likely that the animal is a persistent excreter of organisms. Whey is

prepared by separating the cream from a milk sample by centrifugation and adding rennet to it. The mixture is incubated and the Whey separated. The test is carried out as in SAT.

ELISA method has been described. It is sensitive and specific.

**Goats and Sheep:** Two live vaccines are used to immunize sheep and goats. They are Rev. 1 and H38, however produces side reactions and hence strain Rev. 1 is preferred. In China, *B. suis* strain 2 live vaccine is used to immunize cattle, sheep, goats and pigs against *Brucella* infections in animals. It is believed to be effective, though it is not widely used outside China.

Vaccination against infections in human is not generally carried out. In Russia, people who are at risk are vaccinated with live vaccine strain 19-BA derived originally from *B.abortus* strain 19.A protein polysaccharide complex derived from the cell wall of *Brucella* smooth forms, has been found effective in humans in Russia.

Prevention should aim at quick disposal of aborted fetuses, and placentae in the farm. Veterinarians should ensure that they clean up after attending to retained placentae or cases of abortion to avoid transmitting infection to other farms.

<b>Species</b>	<b>Primary Host</b>	<b>Secondary Host</b>
<i>B. abortus</i>	Cattle	Man, sheep, horses
<i>B. melitensis</i>	Sheep, goats	Man, Cattle
<i>B. suis</i>	Pigs	Man, dogs

<i>B. ovis</i>	Sheep	--
<i>B. canis</i>	Dogs	Man?
<i>B. neotomae</i>	Desert wood rats	--

## **BORDETELLA, MORAXELLA AND ACINETOBACTER**

**Bordetella:** Organisms of the genus were originally classified in the genus *Haemophilus*. They do not require X and V factors for growth and hence they were subsequently placed in a new genus named after Bordet, who with Gengou in 1906, first isolated the organism causing whooping cough. The important species of the genus are:

<b>Species</b>	<b>Host</b>
<i>Bordetella pertussis</i>	Human
<i>Bordetella parapertussis</i>	Human
<i>Bordetella brochiseptica</i>	Human, Pig, Dog and Cat.
<i>Bordetella avium</i>	Turkey.

The organisms of the genus are morphologically similar to *H. influenzae*, but they are more uniform in size, although pleomorphic and thread-shaped forms may be seen particularly in subcultures. They are Gram-negative, showing a tendency to bipolar staining. They all have absolute requirement for nicotinic acid.

Primary isolation of *B. pertussis* can only be obtained on a complex medium containing blood and glycerol-potato or some modification of it. Unsaturated fatty acids are inhibitory to its growth on media. The other species are less fastidious. *B. parapertussis* produces a brownish –

black colouration on potato medium. The pigment is formed from tyrosine and this differentiates it from *B. pertussis*.

***Bordetella pertussis***: It is the causative agent of whooping cough, a disease of infancy and early childhood. The organism was first cultivated by Bordet and Gengou in 1906 on a solid medium containing glycerinated potato extract and fifty percent (50%) blood.

It is a small non-motile ovoid organism when freshly isolated from an active case of whooping cough. On further subculture on artificial media, the cells become pleomorphic, longer and thread-shaped. The organism is capsulated when examined directly from lesions or when first isolated on suitable media. It is Gram-negative and may occasionally show bipolar staining.

Primary isolation can be obtained only on complex media, such as Bordet – Gengou medium or its modification. A highly selective medium which contains at least thirty percent (30%) defibrinated horse blood, starch, glycerol, penicillin, M & B 938 (4-4 diamido – diphenylamine – hydrochloride) and sodium fluoride has been described for primary isolation of the organism. Various media have been described for the nutritional requirements of *B. pertussis* and also for growing large quantities of the organism for vaccine production.

It is an aerobic organism which takes 3-6 days to produce good growth on media. The colonies on Bordet-Gengou are smooth, raised, with entire edge and pearly appearance. There is a “fuzzy” zone of haemolysis around the colony when cultured on a blood containing medium.

Variation in antigenic and morphological characters of *B. pertussis* can be produced by alteration of the growth media and continued subculture. On subcultures there is a gradual loss of virulence which is accompanied by marked changes in antigenic structure and other characters. The change is referred to as S----->R (Smooth to Rough) and for *B. pertussis* four phases 1, 2, 3 and 4 are recognized.

Phase 1 is the fully virulent, encapsulated state of the typical organism.

Phase 4 is the avirulent, noncapsulated state.

Phase 2 and 3 are the intermediate states.

Cells of phases 2, 3 and 4 can be converted to phase 1 by serial passages in mice.

**Virulence factors:** They are associated with toxins of the organism.

1. Heat-labile dermonecrotic toxin which is released after rupture of the cell.
2. A heat stable endotoxin.
3. Haemagglutinin, though its relation to virulence is not clear.

The protective antigens are somatic.

**Laboratory diagnosis:** It is based on the isolation and identification of the organism from the respiratory tract. Swab of the posterior pharynx is inoculated on two plates of Bordet-Gengou agar, (or any other suitable medium) one of them containing penicillin or methicillin to inhibit contaminants. Growth occurs in 3-4 days at 37<sup>0</sup>C incubation. The isolate is confirmed by agglutination with pertussis antiserum. Fluorescent antibody technique may be used along with culture of the organism.

**Immunity:** Recovery from whooping cough often results in long lasting immunity. Second attacks are rare. Maternal antibody transfer is insufficient and hence newborn infants are very susceptible to infection. Vaccination confers substantial protection against whooping cough and the immunity lasts more than 3 years.

***Bordetella parapertussis***: It was first reported by Eldering and Kendrick in 1937. It was isolated from cases of suspected whooping cough. Although it is morphologically indistinguishable from *B. pertussis*, it differs culturally by growing readily on ordinary media and by producing brownish discoloration on media of growth. It grows well on Bordet-Gengou medium producing in twenty four hours colonies which resemble those of *B. pertussis*, but they are large and with the haemolytic zone more darkly discoloured. It is strongly catalase positive.

***Bordetella bronchiseptica***: The organisms are Gram-negative rods or coccobacilli. They were originally isolated from the respiratory tracts of dogs in early distemper infection. They were thought to be the causative agents of the infection, but later a virus was shown to be the primary agent. The organism has also been associated with some infections of humans, pigs, guinea pigs, monkeys, rabbits and cats.

*B. bronchiseptica* is strictly aerobic, motile and it grows on ordinary media. It is haemolytic on blood agar, oxidase, catalase positive and strongly urease positive (usually in 4 hours). It is resistant to nitrofurans which are useful in the preparation of a selective medium.

#### **Virulence factors:**

- (1) It attaches to ciliated epithelium of the respiratory tract by means of pili thereby causing ciliostasis.
- (2) It produces cytotoxins, which are not well characterized.

#### **Diseases**

**Fig:** *B. bronchiseptica* and toxigenic *Pasteurella multocida* type D are the primary agents of swine atrophic rhinitis. The disease is characterized by inflammation and atrophy of nasal turbinates. It is an economically important swine disease.

The severity of infection varies with age (1-8 weeks). The younger the animal the more severe is the infection as illustrated below.

<i>Age Infection</i>	<i>Severe atrophic rhinitis</i>	<i>Mild atrophic rhinitis</i>	<i>Rhinitis only</i>
<i>Less than 3 weeks</i>	+	+	+
<i>3-8 weeks</i>	-	+	+
<b>More than 8 weeks</b>	-	-	+

Transmission is by droplet aerosols from symptomatic infected and asymptomatic carriers to non-infected animals.

### **Pathogenicity**

- (1) **Virulent** *B. bronchiseptica* with or without *P. multocida* type D is transmitted from an infected sow to young pigs via droplet.
- (2) *B. bronchiseptica* organisms attach to the surface of the nasal ciliated epithelial cells using attachment pili for 3-5 weeks.
- (3) They produce cytotoxin that causes loss of ciliated epithelium.
- (4) Both *B. bronchiseptica* and *P. multocida* type D, especially the latter, release dermonecrotic toxins or other toxic factors which prevent the development of the turbinate (turbinate hypoplasia) by some as yet unknown mechanism. There is degeneration of osteoblasts. Atrophy also occurs. Dermonecrotic toxin *per se* has been shown capable of inducing atrophy.

(5) The damage is much potentiated by concurrent infection with toxigenic *P. multocida* type D.

(6) Environmental factors and possibly infections by other bacteria may also potentiate the damage.

Older pigs are resistant to the toxins probably because the ossifying tissues are no longer sensitive to the toxins.

Infected pigs fail to do well. They are prone to other bacterial respiratory infections, for example, *P. multocida* type A, *Mycoplasma sp*, *H. parasuis* and *B. bronchiseptica* itself, which usually result in pneumonia.

**Laboratory diagnosis:** Isolation of *B. bronchiseptica* and *P. multocida* type D from nasal swabs of infected animals.

**Dogs:** *B. bronchiseptica* is associated with kennel cough of dogs. It is a low-grade tracheobronchitis accompanied by rhinitis and persistent cough.

Incubation period is 2-10 days exposure. The duration of coughing is a few days to 2 weeks.

*B. bronchiseptica* is associated with bronchitis and pneumonia often secondary to distemper virus or other respiratory diseases, for example, canine adenovirus and parainfluenza virus.

**Laboratory diagnosis:** Tracheal washing is best for isolation of the organism. Usually it is the only isolate in tracheobronchitis. Mixed bacteria may be isolated in cases of bronchitis or pneumonia.

**Cat:** *B. bronchiseptica* is often isolated from the respiratory tract of clinically normal cats. It is occasionally associated with bronchopneumonia.

**Rabbit:** It may cause tracheobronchitis and rhinitis in rabbits.

**Horse:** Infection with *B. bronchiseptica* is rare in horses. When it occurs, it is associated with respiratory disease.

**Humans:** Cases of human infections have been mainly associated with immunocompromised persons and contact with animals. The infections are often tracheobronchitis, pneumonia and endocarditis. These cases are few.

***Bordetella avium:*** The organism was formerly named *Alcaligenes faecalis*. It causes turkey coryza, a severe rhinotracheitis of young poults. It may predispose infected animals to other infectious agents like *E. coli*. The infection is characterized by sneezing, dyspnoea, oculonasal discharge, tracheal collapse and low growth rate.

#### Characteristics of *Bordetella* species

Characteristics	<i>B. pertussis</i>	<i>B. para-Pertussis</i>	<i>B. bronchiseptica</i>	<i>B. avium</i>
Catalase production	d	+	+	+
Inhibition of growth by fatty acids	+	-	-	-
Motility	-	-	+	+
Growth on MacConkey agar	-	+	+	+
Growth on Bordet-Gengou Medium				
1-2 days	-	+	+	+
3-6 days	+	+	+	+
Nitrate reduction	-	-	+	+
Citrate utilization	-	+	+	+

Urease production	-	+	+a	+
Pertussigen (pertussis toxin)	+	-	-	-
HLT (Heat labile toxin)	+	+	+	---

+: Positive  
-: Negative

d: Strain variation  
a: Positive in 4 hours

-----Doubtful

### ***Moraxella:***

It was formerly referred to as Diplobacillus Morax-Axenfeld. Organisms of the genus are bacilli or coccobacilli, usually in pairs. They show considerable variation in size and shape. They are positive. They grow on simple media containing blood. They do not ferment carbohydrates. They are oxidase and catalase positive. Different species may liquefy gelatin and coagulated serum.

The important species are:

<b>Species</b>	<b>Host</b>	<b>Disease</b>
<i>M. lacunata</i>	Human	Conjunctivitis
<i>M. liquefaciens</i>	Human	Corneal ulceration
<i>M. bovis</i>	Cattle, goat	Kerato conjunctivitis ("pink eye")
<i>M. anatipestifer</i>	Duck	Septicaemia and serositis

(formerly *Pasteurella*)

<i>M. catarrhalis</i>	Human, Cattle, sheep & dog	Bronchitis, Pneumonia Commensal
<i>M. osloensis</i>	Human	Commensal of genital tract

***M. lacunata*:** The organism was described independently by Morax in 1896 and Axenfeld in 1897 (Diplobacillus Morax – Axenfeld) as a Gram-negative bacillus occurring in the pus from conjunctival and Corneal infections of humans. In smears of conjunctival pus it occurs as single bacilli, diplobacilli or in short chains. In subcultures pleomorphic forms of different sizes and shapes may be seen.

Primary isolation of *M. lacunata* is difficult, even on rich media. For primary cultures Loeffler's medium, Filde's medium and Dorset egg medium are very good. Colonies produce "pits" of liquefaction on Loeffler's or Dorset egg medium. The organism can be preserved on the media for weeks at 35<sup>0</sup>C.

*M. lacunata* is also associated with angular blepharocconjunctivitis in humans. In volunteers, a drop of culture instilled into the conjunctival sac, gives rise to a typical conjunctivitis in about five days.

***M. liquefaciens*:** The organism was isolated by Pettit in 1900 from cases of conjunctivitis associated with corneal ulceration in humans. The organism may be an invasive variant of *M. lacunata*. It liquefies gelatin and digests coagulated serum more rapidly than *M. lacunata* as its name suggests.

***M. bovis***: It is the most important animal species affecting cattle and to a less extent goats. It is an opportunistic pathogen. It was isolated by Jones and Little in 1923 from cattle suffering from keratitis and conjunctivitis.

The organism is a Gram-negative plump coccobacillus often in pairs (diplobacillus). Pleomorphism is pronounced in cultures. Freshly isolated organisms from lesions are capsulated. It is an obligate parasite of the eye of cattle transmitted directly or via flies from carriers to other animals. It is a cause of infectious bovine kerato conjunctivitis, “pink eye”. It is highly contagious and an important disease of beef cattle.

**Virulence factors:** They are not fully identified but the following may be associated with virulence, fimbriae or pili, haemolysin, fibrinolysin and dermonecrotic factors.

### **Symptoms**

1. Early clinical disease is characterized by a reddening of the conjunctiva and increased lacrimation.
2. There is swelling, photophobia and pain.
3. The eye may be closed.
4. The cornea becomes opaque and may rupture if it is not treated.

**Laboratory diagnosis:** Eye swab is cultured on blood agar aerobically at 37°C. Virulent *M. bovis* is usually  $\beta$ -haemolytic and may pit the agar.

**Goat:** In goats complete opacity of the cornea usually occurs. Other symptoms as in cattle may be seen in goat infection. Caprine cases respond very well to ointment of penicillin applied to the affected eyes.

***Acinetobacter*:**

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

(1) *A. calcoeceticus* and *A. Iwoffii*.

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

### ***HAEMOPHILUS* (PASTURELLACEAE)**

The family pasturellaceae contains the genera *Actinobacillus*, *Gallibacterium*, *Haemophilus*, *Lonepinella*, *Mannheimia*, *Pasteurella*, and *Phocoenobacter*. All but *Lonepinella* and *Gallibacterium* contain species that are of medical importance.

Members of the genus *Haemophilus*, beyond sharing the family traits of the family *Pasteurellaceae*, 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.

Discussed in this note are *Haemophilus paragallinarum*, (the cause of infectious coryza in chickens) *H. parasuis*, (the cause of a septicemic disease called Glasser's disease or polyserositis, and secondary 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 known as "*Haemophilus somnus*" "*Haemophilus agni*", "*Histophilus ovis*."

All the members of the family *Pasteurellaceae* are gram-negative coccobacilli. They are facultative anaerobes, and typically oxidase-positive (differentiating them from members of the family *Enterobacteriaceae*), most are commensal parasites of animals.

#### **Descriptive Feature**

##### ***Morphology and Staining***

Members of the genera *Haemophilus* and *Histophilus* are gram-negative rods, less than a micrometer wide and 1 to 3 um long, but sometime form longer 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*.

### **Cellular Products of Medical Interest**

**Adhesins.** The role of adhesions, as in other micro-organisms, is to allow the bacterium expressing them 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). Some and probably all members of the family Pasteurellaceae express adhesions (and possibly more than one kind).

*Histophilus somni* also produces a particular surface protein, appearing as fibrils when viewed with an electron microscope. These structures are responsible for the binding of the microorganism to endothelial cells (in which apoptosis is triggered with subsequent fibrin deposition). The protein comprising this fibrillar network is one of two immunoglobulin-binding proteins (IgBPs) produced by *Histophilus somni*-in particular, the so-called high molecular weight IgBP.

**Capsules.** The capsule is involved in many interactions, the most important of which is interference with phagocytosis (antiphagocytic) and involves prevention of deposition of membrane attack complexes generated by the activation of the complement system.

*Haemophilus influenzae* and *H. paragallinarum* produce capsules.

**Cell wall.** Lipopolysaccharide (LPS) elicits an inflammatory response following binding to lipopolysaccharide binding protein (a serum protein), which in turn transfers it to the blood phase of CD14. The CD14-LPS complex binds to Toll-like receptor proteins on the surface of macrophage cells triggering the release of pro-inflammatory cytokines.

The cell wall lipopolysaccharide of *Histophilus somni* is termed lipooligosaccharide (LOS). LOS under the direction of the gene *lob* (for LOS biosynthesis), undergoes phase variation resulting in different epitope expression subsequent to changes in the carbohydrate portions of the *LOS*.

**Iron Acquisition.** Because iron is an absolute growth requirement, microorganisms must acquire this substance if they are to exist within the host. *Haemophili* and *Histophilus* bind transferrin-iron complexes by virtue of iron-regulated outer membrane proteins expressed under iron poor

conditions (so-called transferring binding proteins, or Tbps). Iron is acquired from the transferring-iron complexes that bind to the surface of the microorganism.

### **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. On proper media, members of this genus produce within 24 to 48 hours turbidity in broth and colonies 1 mm in diameter on agar (35-37 °C). 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 *Histophilus* of animals are oxidase and nitrates-positive and ferment carbohydrates.

### **Variability**

Serotype may differ in pathogenicity and geographic prevalence, and determine the specificity of bacteria induced immunity. There are three serotypes (A-C in the so-called Page scheme, or I-III in the Kume scheme) of *H. paragallinarum* and at least seven of *H. parasuis*. Since NAD-dependency is used in the cultural diagnosis of Infectious Coryza, this has become a serious issue.

### **Transmission**

Transmission of *Haemophilus* and *Histophilus* is probably airborne or by close contact, indirect transmission is likely during epidemics.

### **Disease Patterns**

Cattle: Thromboembolic Meningoencephalitis. TEME (Thromboembolic meningoencephalitis) is a consequence of septicemia produced by *Histophilus somni* leading to thrombotic meningoencephalitis and infarcts in brain and cerebellum. The pre-encephalitic stage is marked by high fever. With central nervous system (CNS) involvement, motor and behavioural abnormalities develop.

Pneumonia. *Histophilus somni* occurs in pneumonic processes, usually with other agents, for example *Pasteurella/Mannheimia* spp. As part of the shipping fever complex.

*Septicemia Disease.* *Histophilus somni* may produce septicemia (Manifesting as an endotoxemia), sometimes resulting in arthritis, myocarditis, or abortion.

*Abortion.* Abortion due to *Histophilus somni* sometimes occurs. Whether this is secondary to septicemia, or to genital tract disease (Initiated by an ascending route) is unknown.

*Swine: Pneumonia.* *Haemophilus parasuis* can cause bronchopneumonia secondary to virus infections (e.g swine influenza). Other bacteria (e.g *Pasterurella* spp. and *Mycoplasma spp*) May also participate.

*Septicemic Disease:* In young weaned pigs, *H. parasuis* also causes Glasser's disease (polyserositis), an acute inflammation affecting pleura, peritoneum, mediastinum, pericardium, joints and meninges. Weaning, transport, and management stress are predisposing causes. The disease strikes sporadically within days of the stressing event. Morbidity and mortality are often low because of wide-spread acquired resistance, but they may be high in previously unexposed herds (e.g specific pathogen-free herds). Disease manifestations include fever and general malaise, respiratory and abdominal distress, lameness, and paralytic or convulsive signs. Recovery begins in 1 to 2 weeks. Similar syndromes are due to *Mycoplasma hyorthinis*.

*Poultry: Infectious coryza.* Infectious coryza (caused by *H.paragallinarum*) is an acute contagious upper-respiratory infection of chickens. It affects birds of practically all ages. The sign include nasal discharge, swelling of sinuses, facial oedema, and conjunctivitis. With air sac and lung involvement, rales may be detected. In the uncomplicated infection, mortality is low. Loss of productivity is the most damaging aspect. Superimposed infections with mycoplasmas and helminth parasites exacerbates and prolong outbreaks. Of other species, only Japanese quail are highly susceptible.

## Laboratory Diagnosis

### *Specimens*

*Haemophilus* species are fragile and the specimens should be protected from drying and cultured as soon as possible (within 24 hours) after collection. Refrigeration and transport media do not appear to be beneficial and deep freezing, below  $-60^{\circ}\text{C}$ , is the only definite method for the preservation of these bacteria. The type of specimen required will depend on the disease or lesions present.

### *Direct microscopy*

Demonstration of these small Gram-negative rods in tissues is often difficult and specific fluorescent antibody staining is a sensitive and specific method.

### *Isolation*

X and V factors must be supplied for all the *Haemophilus* species except *H. somnus*. The X factor (haemin) is heat-stable and present in adequate amounts in 5 per cent blood agar. V factor is present mainly intracellularly in red cells and is susceptible to NADases present in most bloods. In chocolate agar the V factor is released from the red cells, the NADases are destroyed, and the heat-stable X factor is still present. *Staphylococcus aureus* grown as a streak across a blood agar plate will provide the V factor. V-factor-requiring haemophili will grow as satellite colonies near the streak. Commercially available media, with supplements, are available for *Haemophilus* species but chocolate agar is the most satisfactory medium for the haemophili isolated from animals. Selective media have been designed for *H. somnus* but their performance has not been consistently successful. The

## Identification

### **Colonial morphology**

Small dewdrop-like colonies may appear after 24-48 hours' incubation and none are consistently haemolytic. A few strains of *H. somnus* may show a frank clearing around the colonies especially on Columbia-base sheep blood agar. *H. somnus* colonies may appear yellowish especially in a loopful of growth or on a confluent lawn.

### **Microscopic appearance**

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

### **Biochemical reactions**

In non specialist laboratories, a presumptive identification of the fastidious Haemophilus species is based on hosta species, clinical signs and lesions, colonial and microscopic characteristics, X and V factor requirements, oxidase and catalase reactions and whether or not CO<sub>2</sub> enhances growth. *H. somnus* is rather variable in biochemical activities and the most reliable reactions are oxidase-positive, catalase-negative with CO<sub>2</sub> giving a considerable enhancement of growth. If the indole test is positive, this is usually diagnostically.

### **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 commercial discs impregnated with V factor, X factor and XV factors, respectively, are placed on a lawn of the test bacterium on a trptose agar plate. Colonies will cluster around the disc(s) supplying the required growth factor(s). However, the results of this test are in 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 is the most satisfactory method for testing the requirement for the X factor. A loopful of growth from young culture is suspended in 0.5ml of a 2mM solution of delta-aminolevulinic acid (ALA) hydrochloride and 0.8mM MgSO<sub>4</sub> in 0.1 M phosphate buffer at PH 6.9. It is incubated for at least 4 hours at 37<sup>0</sup>C and exposed to a wood's UV lamp in a dark room. A red fluorescence indicates that porphyrin is present and the X factor is not required. The test is based on X factor-independent strains being able to convert ALA, a porphyrin precursor, to porphyrin (an intermediate in the haemin biosynthetic pathway). Haemin-dependent strains do not have the appropriate enzymes. Filter paper discs impregnated with ALA are available commercially.

### **Serology**

Surveys have shown that antibody to *H. somnus* is widespread in cattle populations. So far there is no test that is used for the diagnosis of clinical cases.

In poultry, antibody to *H. paragallinarum* is demonstrable after 1-2 weeks of infection and can be detected for over a year. Serological procedures are used to identify potential carrier birds; these include slide and tube agglutination tests, agar gel precipitation, latex agglutination and haemagglutination and haemagglutination-inhibition tests.

## **BORDETELLA**

The bordetellae are small, Gram-negative rods that tend to be coccobacilliary. They are strict aerobes and do not attack carbohydrates but derive energy by the oxidation of amino acids. *B. avium* and *B. bronchiseptica* are motile by peritrichous flagella but *B. pertussis* and *B. parapertussis* 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 inhabitants primarily 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, guinea-pigs, rats, horses and possibly other animals. *B. avium* inhabits the respiratory tract of infected poultry, principally turkeys. Mammalian infections are mainly transmitted by aerosols but in turkeys indirect spread can occur via water and litter.

## **Laboratory Diagnosis**

### **Specimens**

Specimens may include nasal swabs, tracheal washings and pneumonic lungs. If nasal swabs are to be taken from animals where the nasal orifice is small, such as in young pigs, dogs and laboratory animals, the narrow gauge, flexible swabs designed for human infants (such as Mini-Tip Culturette swabs) should be used.

### **Direct microscopy**

As the bordetellae are small Gram-negative coccobacilli, smears directly from specimens are not very useful. A fluorescent antibody technique would be useful.

### **Culture**

The routine media used are sheep blood and MacConkey agars. *B. avium* and *B. bronchiseptica* grow well on both media. The plates are incubated aerobically at 37<sup>0</sup> C for 24-48hours.

If solutions are to be attempted from specimens containing a large number of bacterial contaminants, such as nasal swabs, a selective medium is required. The reason is two-fold to prevent overgrowth and to maintain the alkaline to neutral conditions for the bordetellae. Even a few fermentative bacteria on a medium containing carbohydrates can produce sufficient acid to inhibit the *Bordetella* species.

Several selective media have been described, such as 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 but Smith- Baskerville (SB) medium gives a high isolation rate and is also an indicator medium. The SB medium was designed for the isolation of *B. bronchiseptica* from pigs. If it is used for the isolation of strains from dogs or rabbits, the gentamycin should be omitted as some isolates have been reported as being gentamycin-susceptible. *B.avium* will grow well on SB medium, with or without the antibiotic supplement. The inoculated SB medium is incubated aerobically at 37<sup>0</sup> C for 48 hours.

### **Identification**

#### **Colonial morphology**

On sheep or horse blood agar *B. bronchiseptica* forms very small, convex, smooth colonies with an entire edge after 24 hours. Some strains may be haemolytic. The colonies of *B. avium* are similar but are non-haemolytic. Phase modulation occurs in both species and this is thought to be due to loss of a capsule-like structure on subculture. The virulent, encapsulated phase I colonies

are convex and shiny, those of phase II are larger, circular and convex with a smooth surface and the avirulent phase III colonies are large, flat and granular with an irregular edge.

The colonies on MacConkey agar are small, pale with a pinkish hue and amber discolouration of the underlying medium. *B. avium* and *B. bronchiseptica* have colonies of similar appearance on MacConkey agar.

Smith-Baskerville (SB) medium contains the PH indicator bromothymol blue and the agar is green at PH 6.8. The colonies of *B. avium* and *B. bronchiseptica*, after 24 hours incubation, are small (0.5 mm diameter or less), blue colonies with a lighter blue (alkaline) reaction in the medium around them. After 48 hours' incubation, the colonies are 1.0-2.0 mm diameter, blue or blue with a green centre and the surrounding medium is blue. Any fermentative contaminants give an acid reaction and the colonies and surrounding medium become yellow.

### **Microscopic appearance**

The bordetellae are small Gram-negative coccobacilli.

### **Biochemical reactions**

*B. bronchiseptica* is positive to the oxidase, catalase, citrate, urease and nitrate tests. It is motile and carbohydrates are not utilized. *B. avium* and *Alcaligenes faecalis* have similar reactions to those of *B. bronchiseptica* but are urease-negative and nitrate-negative. *A. faecalis* is present in soil, water and faeces and because of its ubiquity it can occasionally be present as a contaminant in clinical specimens. It has many properties in common with *B. avium* from which it must be distinguished.

### **Haemagglutination test**

*B. bronchiseptica* possesses a haemagglutinin and will haemagglutinate washed sheep erythrocytes. A young 24-hour culture should be used as older colonies tend to lose their haemagglutinating ability. Two colonies of a suspected *B. bronchiseptica* culture are 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 mixed. To check for autoagglutination, controls should include a suspension of colonies without erythrocytes and a suspension of erythrocytes alone. *B. bronchiseptica* will haemagglutinate the red cells within 1-2 minutes.

### **Serology**

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

### **Animal inoculation**

The dermonecrotising toxins of *B. avium* and *B. bronchiseptica* are thought to be important virulence factors. There appears to be no cross-reactivity between the two toxins. They are intracellular, heat-labile toxins. The dermonecrotising toxin of *B. bronchiseptica* is lethal if inoculated intraperitoneally into mice and produces 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.

## **BRUCELLA**

Brucellosis is an infectious bacterial disease caused by members of the genus *Brucella*. It is a disease of worldwide importance and affects a number of animal species. *Brucella* are obligate parasites, requiring an animal host for maintenance. Infections tend to localize in the reticuloendothelial system and genital tract with abortions in females and epididymitis and orchitis in males being the most common clinical manifestations. Chronic infections are common.

DNA-DNA hybridization studies show that *Brucella* is a monospecific genus based on level of overall homology. The traditional species names for the different brucellas continues to be used in a biological species concept system based on host range, in addition to the presence of species-specific markers. The classical nomenclature will be used in this discourse. Additional *Brucella* “species” have recently been described from marine mammals. The different *Brucella* species exhibit host preferences and vary in severity of the disease caused. Dye and phage susceptibility along with biochemical, cultural, and serologic characteristics are used to distinguish among species. The six traditional *Brucella* species are *B. abortus*, *B. canis*, *B. melitensis*, *B. neotomae*, *B. ovis*, and *B. suis*.

### **Descriptive features**

#### ***Morphology and Staining***

Members of the genus *Brucella* are small, gram-negative coccobacilli measuring 0.6 to 1.5  $\mu\text{m}$  by 0.5 to 0.7  $\mu\text{m}$  in size. Cells are fairly uniform and can easily be mistaken for cocci. They are

typically arranged singly but also occur in pairs or dusters. No capsules, flagella, or spores are produced; however, an external envelope has been demonstrated by electron microscopy around *B.abortus*, *B.melitensis* and *B. suis*. *Brucella* stain red with Macchiavello and modified Ziel-Neelsen stains.

### **Cellular Structure and Composition**

Brucellae possess a typically gram-negative cell wall. Dominant surface antigens are located on the lipopolysaccharide. Specifically, the A and M antigens are found in varying concentrations among the different smooth *Brucella* species.

### **Cellular Products of Medical Interest.**

*Cell Wall* - The cell wall of the members of this genus is one typical of gram negatives. In addition, the cell wall LPS of brucellae aid in its survival within macrophage.

*Erythritol*- Erythritol (a four-alcohol) is one of several “allantoic fluid factors” found in the gravid uterus, and appears responsible for the preferential localization to the reproductive tract of the pregnant animals. “Allantoic fluid factors” stimulate the growth of brucellae.

*Outer Membrane Proteins.* Porin proteins in the outer membrane are thought to stimulate delayed-type hypersensitivity and account for the varying susceptibility to dyes observed for the different species.

*Miscellaneous Products.* Production of adenine and guanine monophosphate by *Brucella* inhibit phagolysosome fusion and activation of the myeloperoxidase-halide system. *Brucella* are able to inhibit apoptosis in infected macrophages, thereby preventing host cell elimination. Soluble protein products inhibits TNF-alpha production. The Vir (for virulence) operon encodes a Type IV secretion system, which appear to be involved with intramacrophage survival.

### **Growth Characteristics**

On initial isolation, colonies are not apparent until 3 to 5 days' incubation. Most colonies are detected by 10 to 14 days, but in some cases incubation for up to 21 days is required. Growth is best in an aerobic environment at 37°C but occurs between 20°C and 40°C. Optimal pH is 6.6 to 7.4. *Brucella ovis* and some biovars of *B. abortus* require an increased concentration of CO<sub>2</sub>. Enriched media with 5% serum are required by *B. abortus* biovar 2 and *B.ovis*.

*Brucella* colonies have a characteristic bluish color when examined with obliquely transmitted light. Colonies have smooth or non smooth morphologies that are determined by the presence or

absence, respectively, of the polysaccharide side chain in the lipopolysaccharide. These morphologic variations are the result of spontaneous mutation and are influenced by specific growth factors. Smooth colonies are white, convex with an entire edge, and have a creamy consistency. Non-smooth colonies have intermediate rough, or mucoid forms. Rough colonies are dull yellow, opaque, and friable. They are difficult to suspend in solution and agglutinate spontaneously. The mucoid colonies are similar to the rough colonies except for having a glutinous texture.

### **Diversity**

The *Brucella* genome appears to be very stable. Strains associated with a specific animal host have a unique genomic organization

The colony morphology of *Brucella* varies from rough to smooth forms (as mentioned above). *Brucella abortus*, *B. melitensis*, *B. suis*, and *B. neotomae* are typically isolated in the smooth form but can develop rough forms on subsequent laboratory passage. *Brucella ovis* is always in a rough form. Isolates of *B. canis* have a mucoid appearance. In general, smooth strains of *Brucella* are more virulent than rough strains.

Variation in CO<sub>2</sub> requirement, H<sub>2</sub>S production, urease production, susceptibility to differing concentrations of certain dyes (thionin and basic fuchsin), and susceptibility to naturally or mutagen-derived bacteriophage account for diversity among species and biovars within species. The different species of *Brucella* vary in host preference and degree of virulence within and among animal species.

### **Transmission**

*Brucella* are disseminated by direct or indirect contact with infected animals. Ingestion is the most common route of entry, although exposure through the conjunctival and genital mucosa, skin, and respiratory routes occurs. The major source for exposure to *B. abortus* in cattle and *B. melitensis* in sheep and goats is through aborted fetuses, the placenta, and post-abortion uterine fluids. Aborted tissue and fluids are also a common means for transmission of *B. suis* and *B. canis*. Genital infections in cattle routinely clear within 30 days after calving and cows are not considered infectious for other cattle after that time. Genital infections in swine, in some cases, persist longer than those in cattle. Ingestion of milk from infected cattle and goats is another source for ingestion of calves and kids. Direct transfer in-utero has also been documented.

Infections of the accessory sex glands of males allows for dissemination of organism through the semen. Infections can occur in the accessory sex organs without testicular or epididymal lesions being present. Venereal transmission of *B.suis* in swine, *B.ovis* in sheep, and *B. canis* in dogs is common. Urine is another vehicle for disseminating *B.canis* to other dogs.

Insects may play a minor role in transmission and maintenance of infection in a herd. Face flies have been shown to take up and excrete Brucella in their feces.

## **Laboratory Diagnosis**

### ***Specimens***

Great care should be employed when working with infected tissues and culture in the laboratory. All *Brucella* culture should be handled following biosafety level 3 practices because of the potential for laboratory infection. All laboratory procedures should be performed in a manner that prevents aerosolization, and all work should be conducted in a biological cabinet.

Appropriate samples for diagnosis of brucellosis depend on the animals species affected, species of *Brucella* involved and clinical presentation. Abscess material, semen, and vaginal fluids associated with recent abortion are useful for recovering organisms ante-mortem. Milk samples from cattle and goats are used in ante-mortem isolation attempts and for immunodiagnostic evaluation. In dogs, blood cultures are useful for isolation of *B. canis* because of the prolonged bacteremia that occurs. Serum is used for serologic evaluation.

Samples collected at necropsy should include spleen, liver, udder, and multiple lymph nodes, including the supramammary, retropharyngeal, internal iliac, lumbar, and mesenteric lymph nodes. The supramammary lymph node is superior to other lymph nodes for isolating *Brucella* from dairy cattle. Abomasal fluid and lungs of the aborted fetus and the placenta are the preferred specimens in the case of abortion. In males the epididymis, testicle, and accessory sex organs are examined.

### **Direct Examination**

Gram stains of foetal stomach contents from an aborted foetus and the placenta reveal large numbers of gram-negative coccobacilli. Using carbol fuchsin rather than safranin as a counter-stain in the gram-staining procedure makes organisms more easily detectable. Modified Ziehl-Neelsen and Macchiavello stains are also used to demonstrate *Brucella*. Organisms can be

detected in semen but are usually in low numbers. *Brucella* is difficult to detect by direct examination in other samples, especially from chronically infected animals.

### **Isolation**

Tissues are cultured directly on solid media. Milk cultures are performed by centrifuging milk at 5900 to 7700 x g for 15 minutes or by allowing for gravity cream separation to occur overnight. Both the cream layer and sediment, if the centrifugation technique is used, should be plated on solid media. Commonly used media include serum dextrose, tryptose, and brucella (Albimi) agars.

If contamination is likely to be a problem, isolation attempts should be made using media containing actidione (30 mg/L), bacitracin (7500 U/L), and polymyxin B (1800U/L). Selective media are used both with and without the incorporation of ethyl violet (1:800,000). Cultures should be incubated at 37°C in 10% CO<sub>2</sub> for a minimum of 10 days and up to 21 days in highly suspicious cases.

Animal inoculation is the most sensitive method for detection of *Brucella* and is sometimes necessary when very low numbers of organism are present. Guinea pigs are the most sensitive laboratory animals for this purpose. Two guinea pigs are inoculated and at 3 and 6 weeks post inoculation an animal is sacrificed. Serum is examined for antibodies and tissues are cultured for organism.

### **Identification**

Presumptive identification of *Brucella* species requires demonstrating colonies of gram-negative coccobacilli that are non-haemolytic, catalase positive, and oxidase positive (except for *B. ovis*, and some strains of *B. abortus*). Most species, except *B. ovis* are strongly urease positive. Glucose and lactose are not fermented by any of the species. Agglutination in unabsorbed anti-smooth *Brucella* serum helps in presumptive identification of smooth strains.

Definitive identification is usually performed by a *Brucella* reference laboratory. A fluorescent antibody test is used for rapid identification. Urease production, CO<sub>2</sub> requirement, H<sub>2</sub>S production, oxidation of metabolic substrates, agglutination in monospecific antisera, and growth in the presence of varying concentration of thionin, and basic fuchin and phage typing are used to determine species and biovars within species. *Brucella abortus* strain 19 can be differentiated from field strains of *B. abortus* by its lack of requirement for CO<sub>2</sub> for growth and inhibition by 5

mg/ml penicillin or 1 mg/ml of erythritol. Strain RB51 can be differentiated from field strains and strain 19 by demonstrating its resistance to rifampin (200 µg/ml), staining with crystal violet, and agglutination with acriflavin.

### **Immunodiagnosis**

Antibody detection is commonly used for diagnosing brucellosis and in control programs. Samples tested include blood, milk, and occasionally semen. A number of immunodiagnostic tests have been developed for cattle. These tests detect different classes and types of antibodies and vary in their sensitivity and specificity. Individual blood samples can be tested by tube agglutination, plate agglutination, rose Bengal plate, or card tests. Other tests include the buffered plate agglutination assay, rivanol agglutination, complement fixation, and enzyme-linked immunosorbent assay (ELISA).

Frequently, highly sensitive but less specific tests are used for screening purposes and are followed by more specific tests for confirmation purposes. A similar approach to that used in cattle is employed when testing goats and sheep for *B. melitensis*. Sera are screened with a test such as the rose Bengal test and result confirmed with a more specific test.

Milk is screened with the *Brucella* milk ring test, which identifies specific antibodies in milk. The test is performed on bulk tank milk samples as a means of screening dairy herds. Stained *Brucella* antigen is added to milk. If antibodies are present, agglutinated antigen is buoyed to the top by the rising cream and a purple ring develops at the top of the tube.

Serologic tests are commonly used to identify infected swine herds and monitor herd status. These tests are less accurate when testing individual pigs because some infected swine do not have detectable antibody titers. Herds can be screened by the brucellosis card test. Tests such as rivanol agglutination and 2-mercaptoethanol agglutination are used for confirmation.

Rams are tested for antibodies to *B. ovis* using either a complement fixation test or ELISA.

For canine brucellosis, screening is performed with a rapid slide agglutination test (RSAT). The RSAT is sensitive but not very specific, therefore positive results should be confirmed with additional tests that are more specific. An agar gel immunodiffusion test using cytoplasmic antigen is more specific but not as sensitive as the RSAT and is used as a confirmatory test.

### **Non-culture Detection Methods**

A number of nonculture methods, including PCR, immunoperoxidase staining, DNA probes, and coagglutination, have been described for detection of *Brucella* in tissues and fluids.

### **Taylorella equigenitalis**

Members of the genus *Taylorella* are 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 clinical and economic importance, *T. equigenitalis* will be discussed in detail. Because of its phenotypic similarity to *T. equigenitalis*, *T. asinigenitalis* will be briefly described.

*Taylorella equigenitalis* (formerly *Haemophilus equigenitalis*) is a Gram-negative rod about 0.8 x 5.0 µm in size. It is a facultative anaerobe, non-motile, oxidase-positive, catalase-positive, phosphatase-positive and produces no acid from carbohydrates. *T. equigenitalis* is a fastidious and slow-growing bacterium and optimal growth is obtained on chocolate agar with a rich base (Eugon or Columbia agar) at 37<sup>0</sup> C under 5-10 per cent CO<sub>2</sub>. 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 disease but sexes can remain carriers indefinitely. The disease is highly contagious. The disease is most common in Europe, Japan, Australia and USA.

#### **Pathogenicity**

Transmission is essentially venereal, but the mares can also be infected by attendants and via instruments. The organisms can be isolated from neonatal and virgin animals. A purulent metritis develops within a few days infection and the mare often has a copious mucopurulent uterine discharge. The infectious process is limited to the mucous membranes of the uterus, cervix and vagina. There is erosion and degenerative change in the endometrium. After endometrial repair is complete, within a few weeks, the organism may still be present in the clitoral sinuses and fossa and can remain there for long periods. No clinical signs occur in the stallion but *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**

In many countries contagious equine metritis is controlled by the Dept. of Agriculture or by the Thoroughbred Breeders' Association. These bodies lay down the method of sample taking, the type of samples to be taken and the culture media to be used, when examining asymptomatic stallions and mares. The requirements may be amended periodically. Often only approved laboratories are licensed to process and culture the specimens. In general, acceptable samples are swabs or biopsies from :

- Mares: cervix, uterus, clitoral fossa and clitoral sinuses.
- Stallions: urethra, urethral fossa and diverticulum, prepuce and pre-ejaculatory fluid.

In some cases it is specified that a stallion serves two maiden mares and these are sampled instead of the stallion. The specimens are collected on sterile swabs and these are placed into Amies transport medium with charcoal. They reach the laboratory, under refrigeration, within 48 hours of collection.

### **Direct microscopy**

Gram-stained smears are of use only on uterine exudates from a mare with clinical disease. *T. equigenitalis* can appear as Gram-negative rods, coccobacilli or short-filaments.

### **Isolation**

Routine medium is chocolate agar with a highly nutritive base such as Eugon or Columbia agar and preferably equine blood. The inoculated plates are incubated at 37<sup>0</sup> C under 10 per cent CO<sub>2</sub>. Growth may be seen at 48hours but negative plates should be examined daily for up to 7 days before discarding them. However, 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**

After 48 hours' incubation the colonies are under 1mm in diameter, shiny, smooth and grayish-white. They may attain a size of 1.5mm on further incubation.

### **Microscopic appearance**

Gram-negative pleomorphic coccobacilli are seen in smears from the colonies.

### **Biochemical reactions**

Colonies with the correct macroscopic and microscopic appearance that are catalase-positive and oxidase positive 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<sup>0</sup>C for up to 2 hours. A yellow colour indicates a positive result.

### **Serology**

Complement-fixing antibodies are consistently detectable from the third to seventh week post-infection in mares. However, this is rather late for the test to be diagnostically and CFT titres do not correlate sufficiently well with the carrier state. Demonstration of CFT antibodies may be useful, in retrospect, to confirm a past infection.

### **Antibodies susceptibility**

Conventional antibiotic susceptibility tests are difficult with this slow-growing, fastidious bacterium. Topical treatment of the clitoral fossa in mares and external genitalia of stallions, on 5 consecutive days, with 2 per cent chlorhexidine followed by 0.2 per cent nitrofurazone ointment is often used.