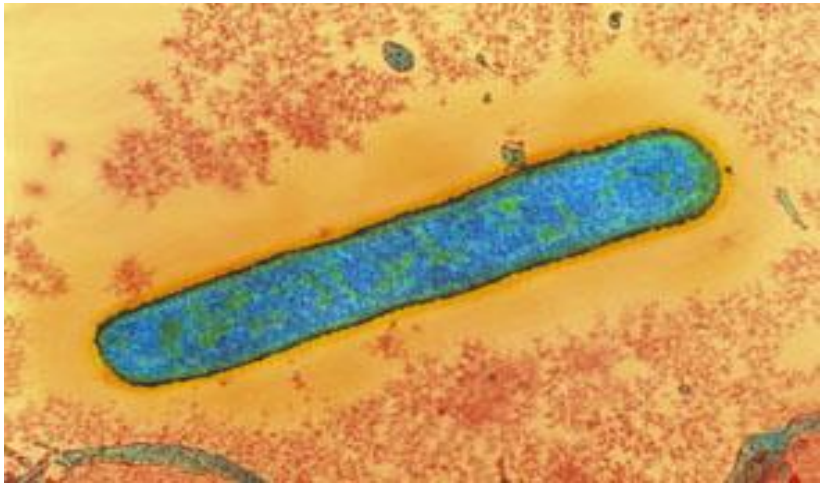
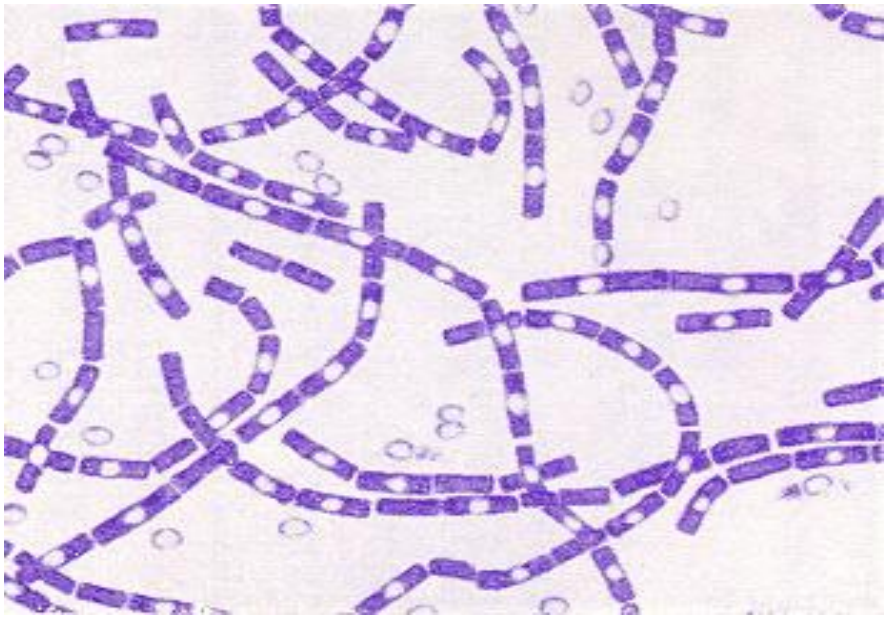
Bacillus anthracis



Bacillus anthracis

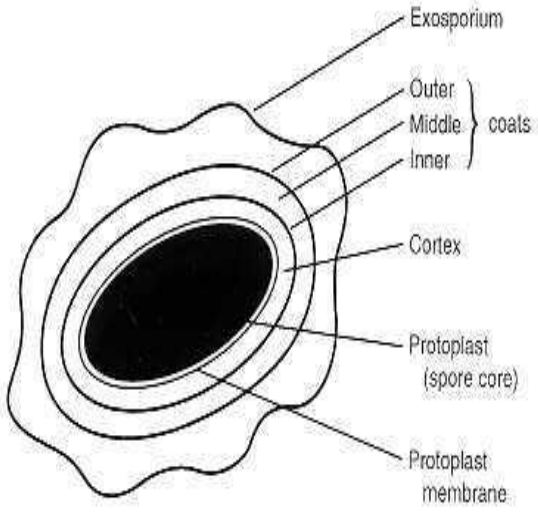
- **Gram + rod**
- **Facultative anaerobe**
- **1 - 1.2µm in width x 3 - 5µm in length**
- **Belongs to the *B. cereus* family**
  - **Thiamin growth requirement**
  - **Glutamyl-polypeptide capsule**
  - **Nonmotile**
- **Forms oval, centrally located endospores**



http://www.bact.wisc.edu/Bact330/lectureanthrax

Endospore

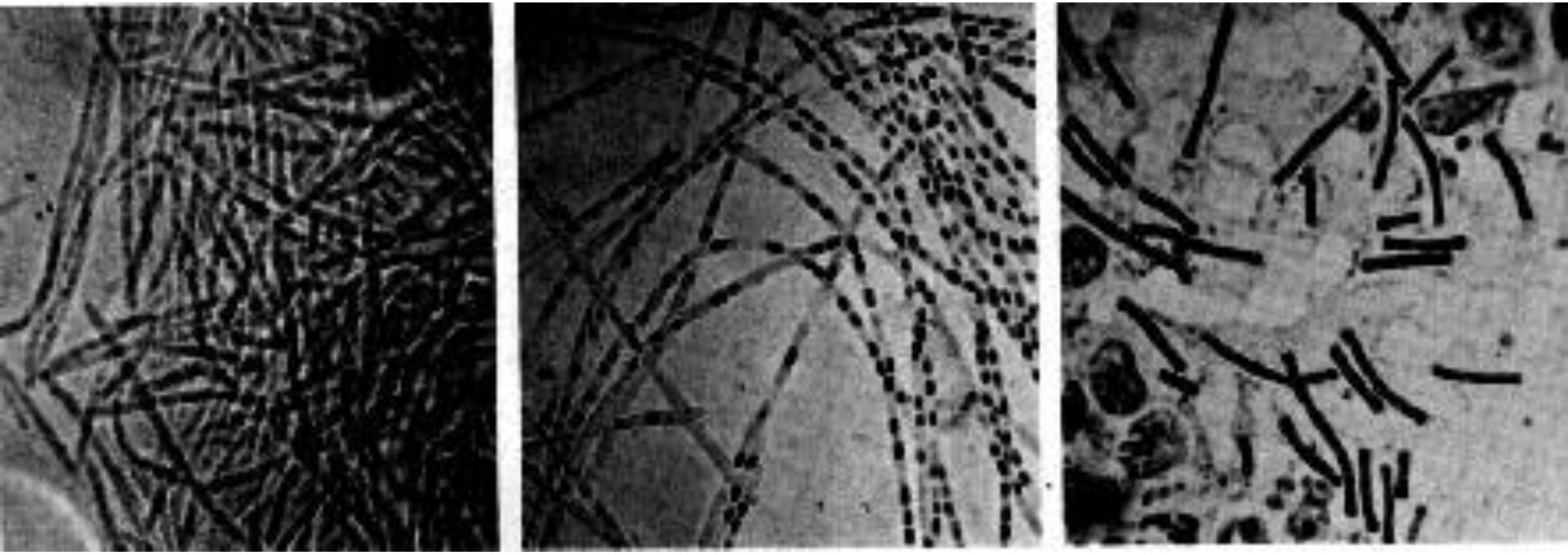
- Oxygen required for sporulation
- 1 spore per cell
- dehydrated cells
  - Highly resistant to heat, cold, chemical disinfectants, dry periods
- Protoplast carries the material for future vegetative cell
- Cortex provides heat and radiation resistance
- Spore wall provides protection from chemicals & enzymes



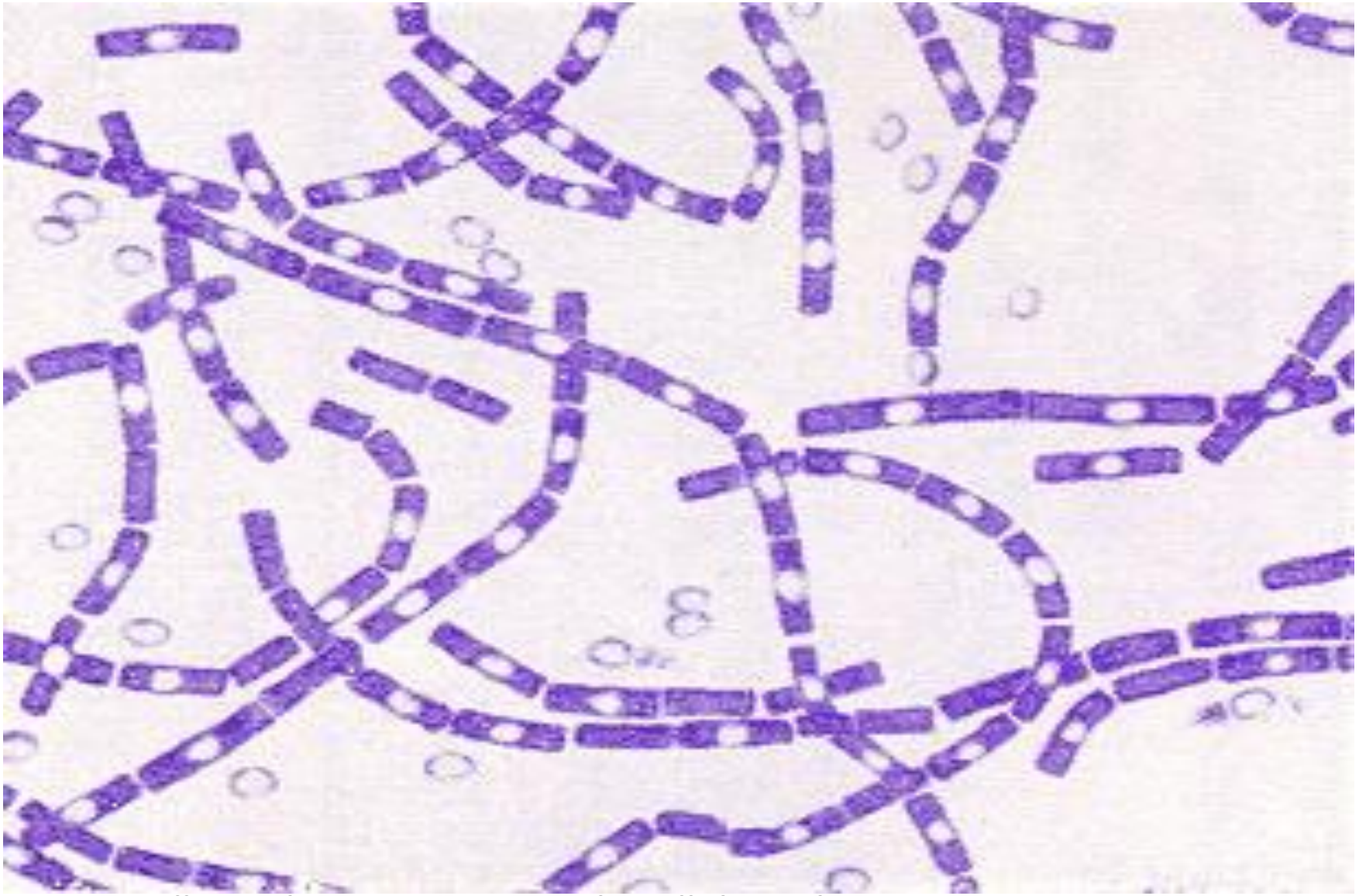
http://www.gsbs.utmb.edu/microbook/ch01

Bacillus anthracis

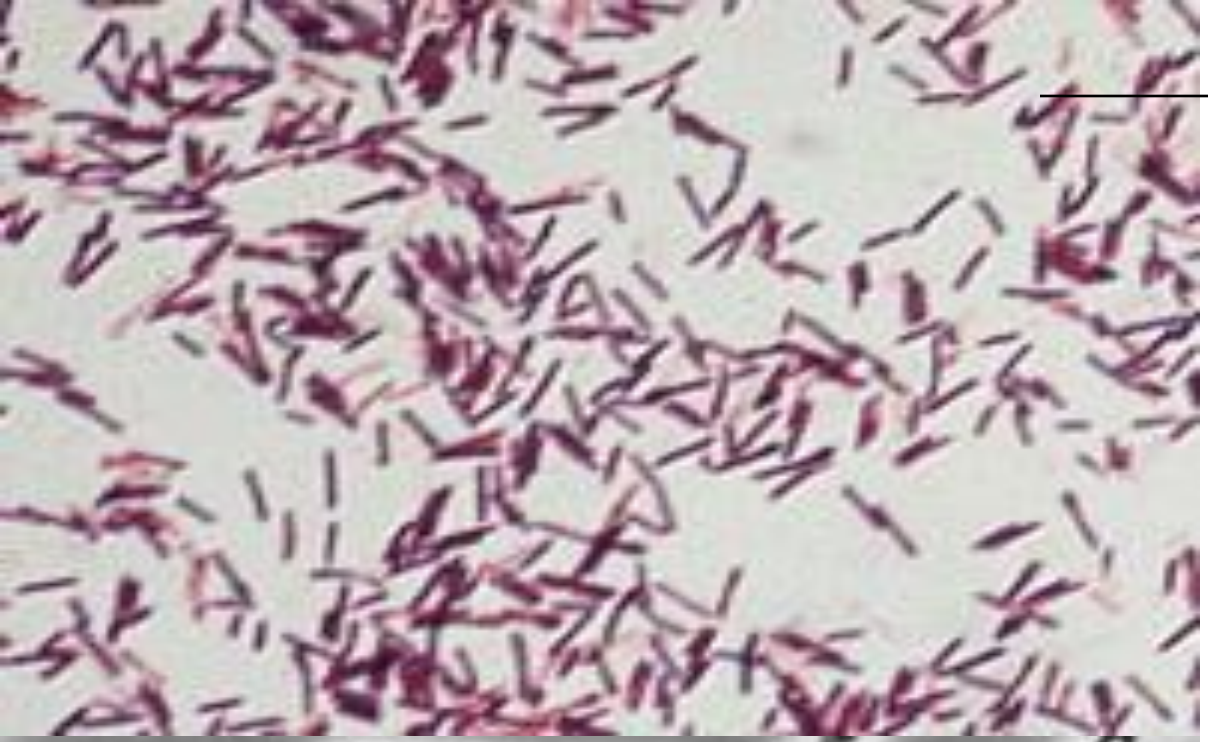
- Gram positive rods
- Capsulated (Protein) – Capsule form in animal tissue and in special laboratory condition (5% CO^2)
- Forms endospore, centrally located, do not form in animal tissues
- MacFadyean (Polychrome methylene blue) stain blue bacilli with purple capsule
- Aerobic/ Facultative anerobe
- Grows on all ordinary medium (Medusa head appearance-uneven wavy margin)
- Inverted fur tree appearance in liquid medium
- Biochemicals : Catalase +, reduces nitrate to nitrite, lecithinase+, glucose, maltose, sucrose, trehalose fermented



Robert Koch's original micrographs of the anthrax bacillus

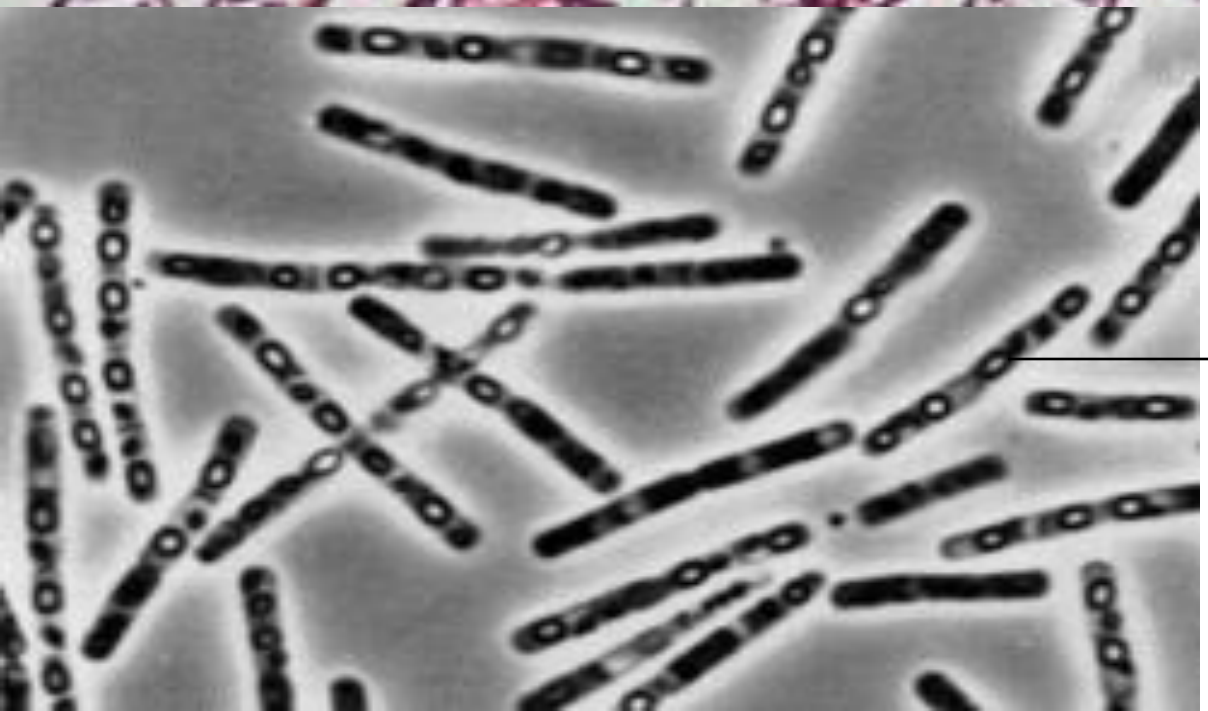


Bacillus anthracis. **Gram stain. The cells have characteristic squared ends. The endospores are ellipsoidal shaped and located centrally in the sporangium. The spores are highly refractile to light and resistant to staining.**

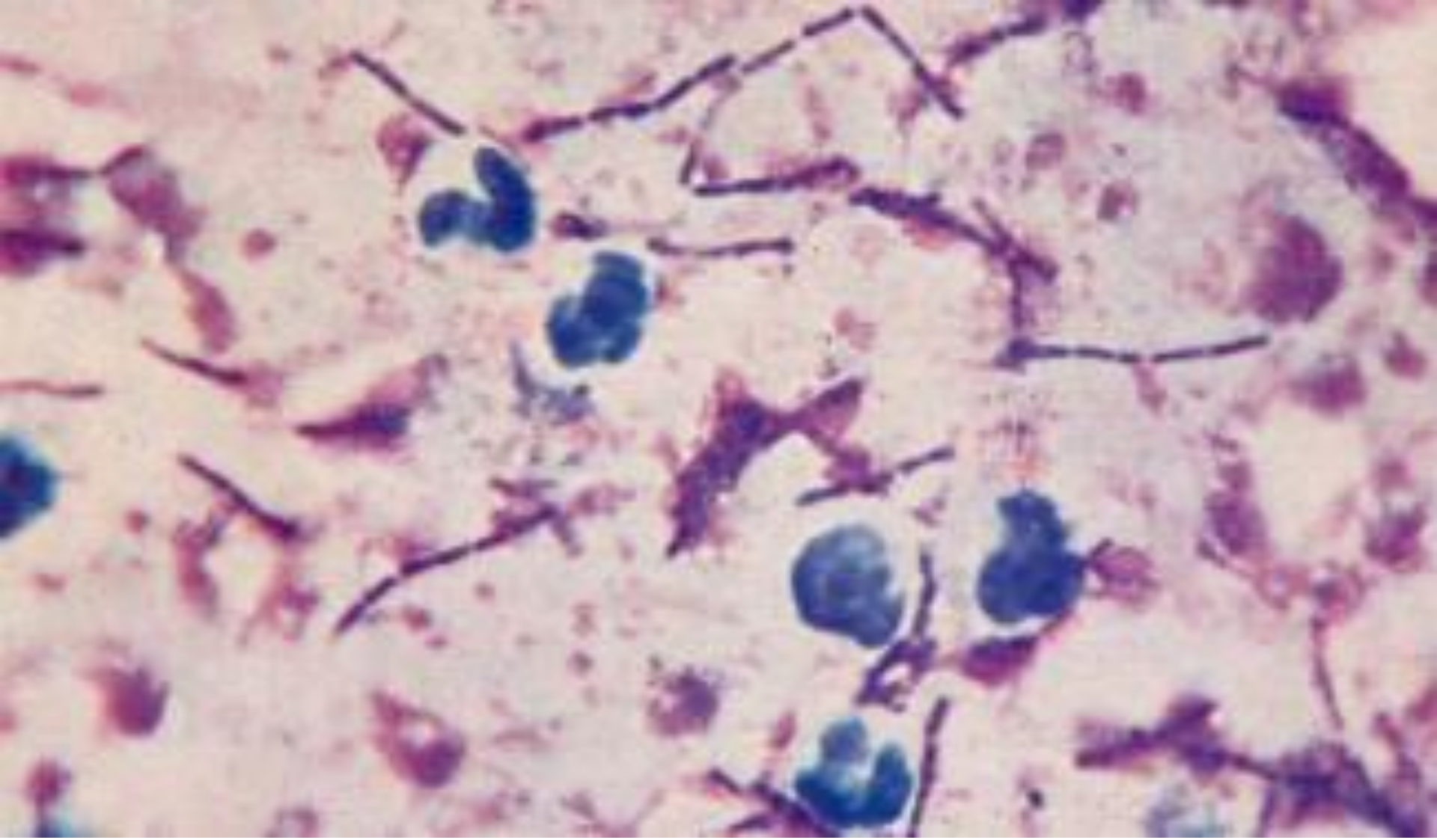


Bacillus cereus

Genotypically and phenotypically it is very similar to *Bacillus cereus,* which is found in soil habitats around the world



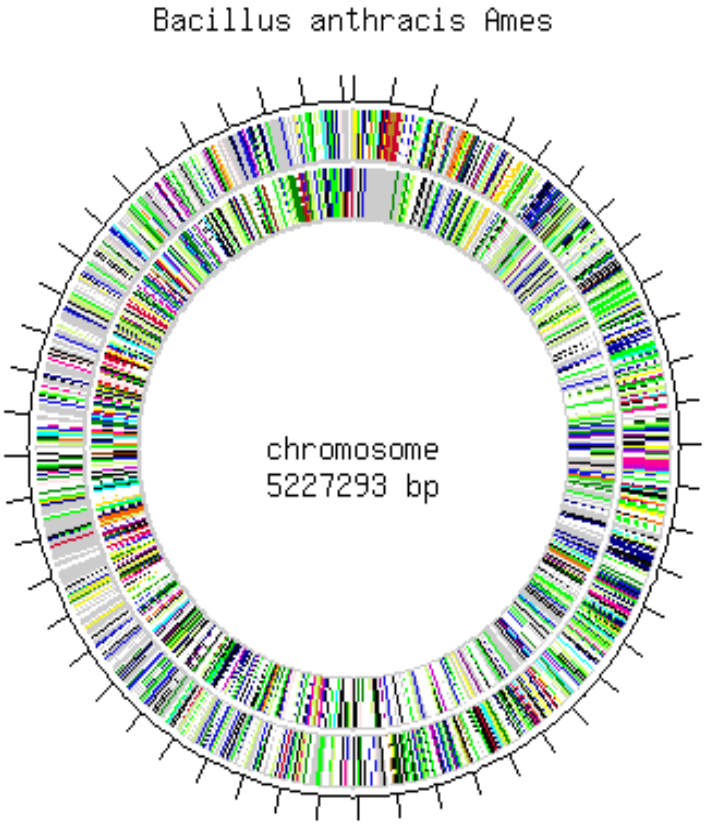
Bacillus thuringiensis. **Phase Photomicrograph of vegetative cells, intracellular spores (light) and parasporal crystals (dark).**



McFadyean's reaction showing short chains of *Bacillus anthracis* cells lying among amorphous,disintegrated capsular material. White blood cells can also be seen.

Genetics

- 1 chromosome
  - 5.2 million bp
  - Ames strain sequenced
- 2 plasmids
  - px01
    - 184 kbp
    - Pathogenicity island
  - pX02
    - 95.3 kbp
    - Capsule
- Anthrax receptor
  - Occurs > than ten thousendfold on macrophage cell
  - ATR/TEM8 gene
    - Chromosome 4



gib.genes.nig.ac.jp/single/ main.php?spid=Bar

Differential Characteristics of *B. anthracis B. cereus and B. thuringiensis*

Characteristic Differential Characteristics of *B. anthracis B. cereus and B. thuringiensis*	*B. anthracis*	*B. cereus* and *B. thuringiensis*
growth requirement for thiamin	+	-
hemolysis on sheep blood agar	-	+
glutamyl-polypeptide capsule	+	-
lysis by gamma phage	+	-
motility	-	+
growth on chloralhydrate agar	-	+
string-of-pearls test	+	-

Table 45. Summary of the differentiating characteristics of the members of the *Bacillus cereus* group.

Tests	*B. anthracis*	*B. cereus*	*B. mycoides*	*B. thuringiensis*
Motility	–	+	–	+
Crystalline, parasporal inclusions (3-day cultures)	–	–	–	+
Haemolysis	– (or weak)	+	weak	+
Penicillin susceptibility (10-unit disc)	S	R	R	R
Gelatin stab culture	'inverted fir tree' type of growth	← rapid liquefaction →		
Lecithinase activity (egg-yolk agar)	+ weak	+	+	+
Nutrient agar with 0.7% Na bicarbonate under 10% CO_2	mucoid colonies	unchanged	unchanged	unchanged
Susceptibility to cherry gamma phage*	+ (lysis)	–	(+) lysis may occur	–
Pathogenicity for mice or guinea-pigs (subcut. or i/v)	+ (death in 24–-48 hours)	+ large dose (non-invasive)	–	–

Physical properties (methods for decontamination)

- SPORES SURVIVE FOR MANY YEARS (DRY STATE AND SOIL)
- Moist heat kills – Vegetative cells 60 ^{0} C X 30 minutes

  Spores 100 ^{0} C X 10 minutes

  4% Formaldehyde kills spores

  4% $KMnO_4$ kills spores

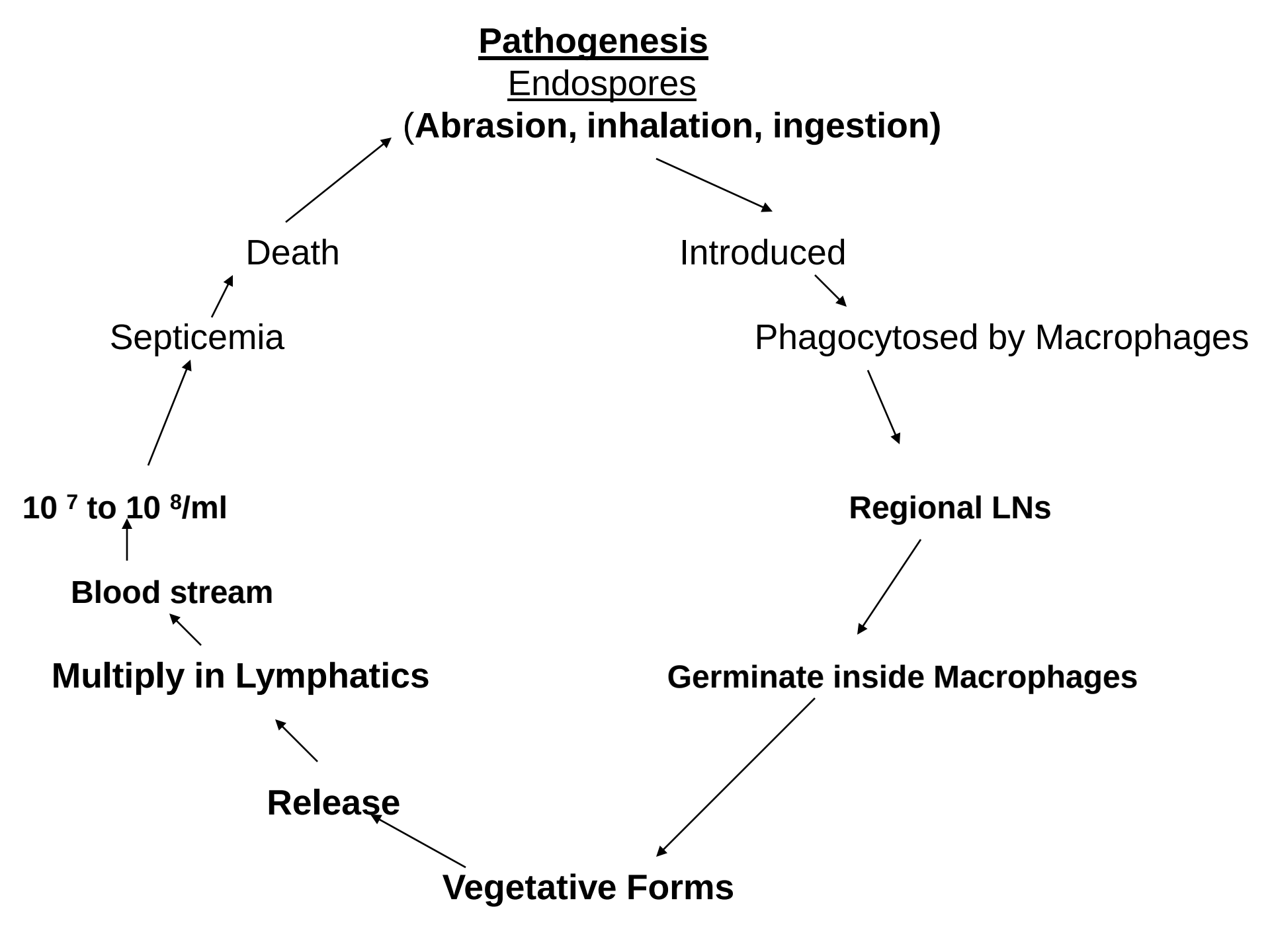
  Hypochlorite (0.5%) commercially available kills spores

Anthrax

- From the Greek word *anthrakos* for coal
- Caused by spores
- Primarily a disease of domesticated & wild animals
  - Herbivores such as sheep, cows, horses, goats
- Natural reservoir is soil
  - Does not depend on an animal reservoir making it hard to eradicate
  - Cannot be regularly cultivated from soils where there is an absence of endemic anthrax
  - Occurs sporadically throughout US
  - South Dakota, Arkansas, Texas, Louisiana, Mississippi, California recognized endemic areas
- Anthrax zones
  - Soil rich in organic matter (pH < 6.0)
  - Dramatic changes in climate

Anthrax Infection & Spread

- **May be spread by streams, insects, wild animals, birds, contaminated wastes**
- **Animals infected by soilborne spores in food & water or bites from certain insects**
- **Humans can be infected when in contact with flesh, bones, hides, hair, & excrement**
  - **nonindustrial or industrial**
  - **cutaneous & inhalational most common**
- **Risk of natural infection 1/100,000**
  - **Outbreaks occur in endemic areas after outbreaks in livestock**


Pathogenesis
Endospores
(Abrasion, inhalation, ingestion)
Introduced
Phagocytosed by Macrophages
Regional LNs
Germinate inside Macrophages
Vegetative Forms
Release
Multiply in Lymphatics
Blood stream
10 7 to 10 8/ml
Septicemia
Death

Three forms of Anthrax

- **Cutaneous** anthrax
  - Skin
  - Most common
  - Spores enter to skin through small lesions
- **Inhalation** anthrax
  - Spores are inhaled
- **Gastrointestinal (GI)** anthrax
  - Spores are ingested
  - Oral-pharyngeal and abdominal

Milestones in Anthrax History

- **Early history**
- **1800s**
- **1900s**
- **Recent years**
- **Outbreaks in Thailand and US**

History of Anthrax (Early history)

- **Although anthrax dates back more than 3,000 years, it was not recognized as a disease until the 18th century.**
- **1500 B.C - A "plague of boils" in Egypt affected the Pharaoh's cattle. 'Boils' are symptomatic of anthrax.**
- **1600s - The "Black Bane" thought to be anthrax, in Europe kills over 60,000 cattle.**
- **1700s - There are some accounts of human cases.**

ANTHRAX

- The anthrax bacillus, ***Bacillus anthracis,*** was the first bacterium shown to be the cause of a disease- **Koch's Postulate**

- In 1877, Robert Koch grew the organism in pure culture, demonstrated its ability to form endospores, and produced experimental anthrax by injecting it into animals.

- Anthrax is a disease of domesticated and wild animals

- Men suffer from anthrax occasionally due to close contact with infected animal or animal products

History (1800s)

- **Early 1800s - The first human cases of cutaneous anthrax in the US and UK were reported in men who contracted the disease after having been in contact with infected livestock.**
- **The disease was called Wool Sorter's disease or Rag Picker's disease because it affected workers in those trades.**
- **1868 - Anthrax was observed under a microscope.**
- **1876 - German bacteriologist Robert Koch confirmed bacterial origin of anthrax.**

History (Early 1900s)

- **1915 - German agents injected horses, mules, and cattle with anthrax during WWI. This was the first recorded use of anthrax as a biological weapon.**
- **1937 - Japan started a biological warfare program in Manchuria, including tests involving anthrax.**
- **1942 - UK demonstrated experiments using anthrax at Gruinard Island off the coast of Scotland.**
- **1943 - United States began developing anthrax weapons.**
- **1945 - In Iran an anthrax outbreak killed more than 1 million sheep.**

History (Late 1900s)

- 1950s and 60s - U.S. biological warfare program continues after WWII at Fort Detrick, Maryland
- 1969 - President Nixon ended United States' offensive biological weapons program, but defensive work still continues.
- 1970 - Anthrax vaccine for humans was approved by U.S. FDA.
- 1978-80 - The world's largest outbreak of human anthrax via insect vectors or contaminated meat struck Zimbabwe, Africa where more than 10,000 cases were recorded and over 180 people died.
- 1979 - In Soviet Union, aerosolized anthrax spores were released accidentally at a military facility, affecting 94 and killing 64 people.

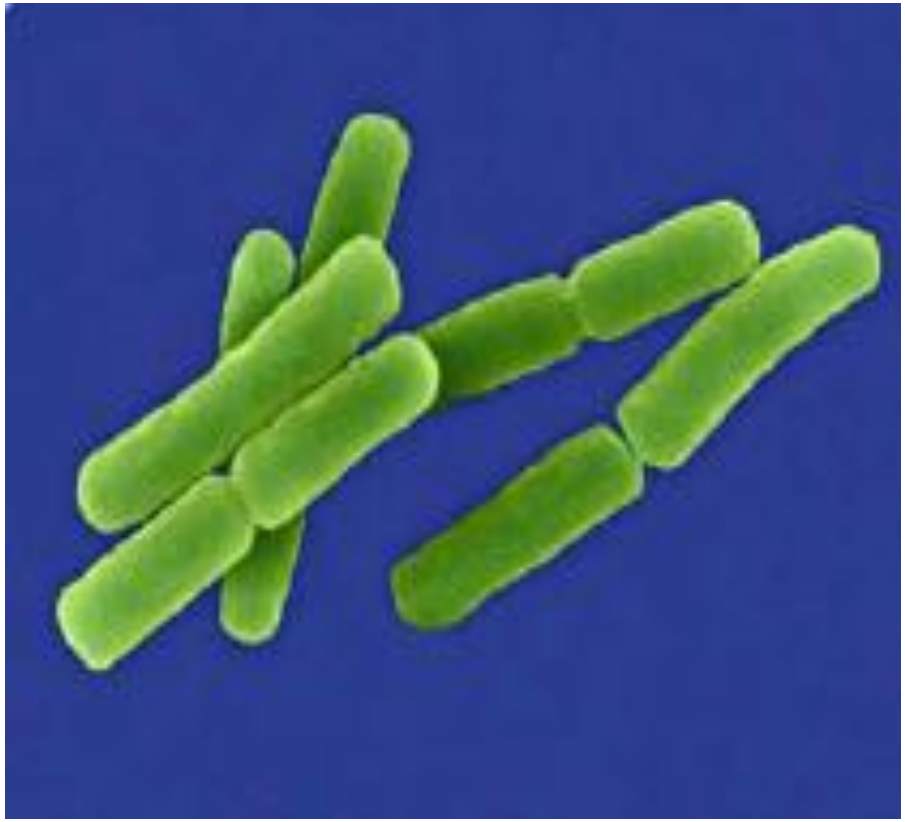
History (Recent years)

- **1991 - About 150,000 U.S. troops were vaccinated for anthrax in preparation for Gulf War.**
- **1990-93 - The cult group, Aum Shinrikyo, released anthrax spores in Tokyo, fortunately no one was injured. On February 27, 2004, the leader of this group was given a sentence of death at a district court in Tokyo.**
- **1995 - Iraq produced 8,500 liters of concentrated anthrax as part of the biological weapon program under Saddam Hussein's administration.**
- **2001 - Letters containing anthrax spores were mailed to many places in the US such as NBC, New York Times, and Media in Miami. In Florida, a man died after inhaling anthrax at the office.**

Pathogenesis

- **The infectious dose of *B. anthracis* in humans by any route is not precisely known.**
  - **Rely on primate data**
  - **Minimum infection dose of ~ 1,000-8,000 spores**
  - **LD_{50} of 8,000-10,000 spores for inhalation**

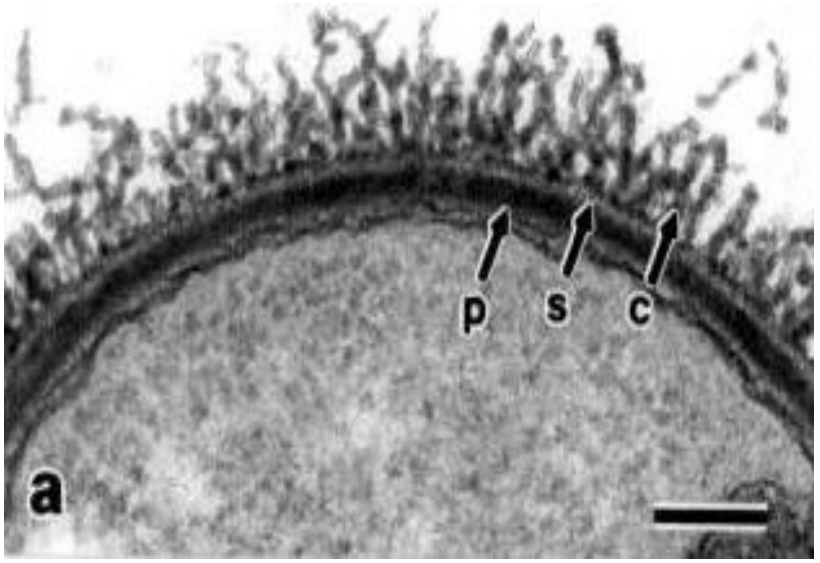
- **Virulence depends on 2 factors**
  - **Capsule**
  - **3 toxins**



http://www.kvarkadabra.net/index.html?/biologija/teksti/biolosko_orozje.htm

Capsule

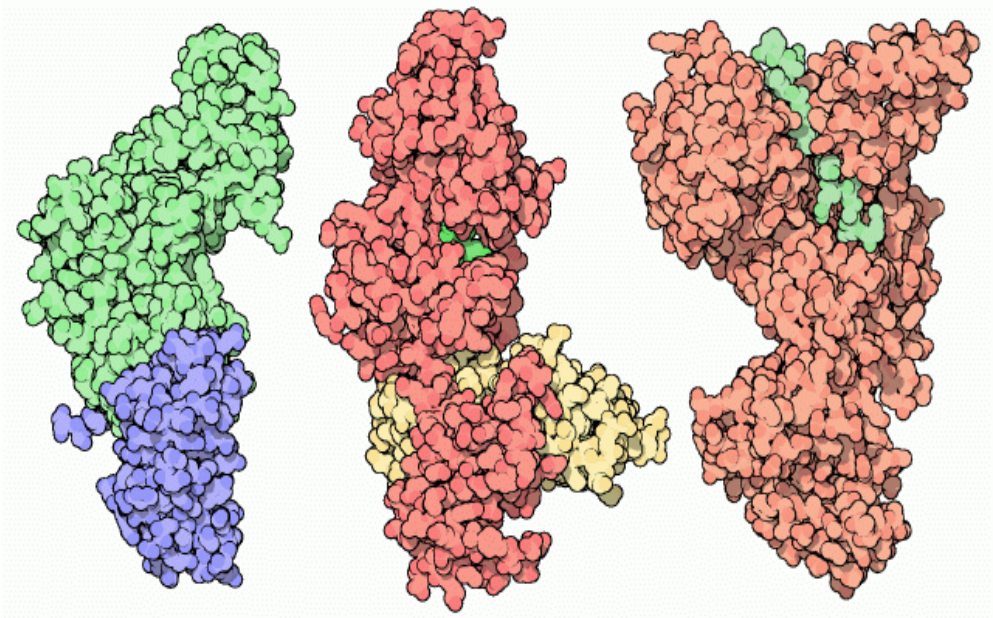
- **Glycocalyx**
  - **Sticky, gelatinous polymer external to cell wall**
- **pX02 plasmid**
- **Made up of D-glutamic acid**
- **Non-toxic on its own**
- **Only encapsulated *B. anthracis* virulent**
- **Most important role during establishment of disease**
  - **Protects against phagocytosis & lysis during vegetative state**



http://textbookofbacteriology.net/BSRP.html

Toxins

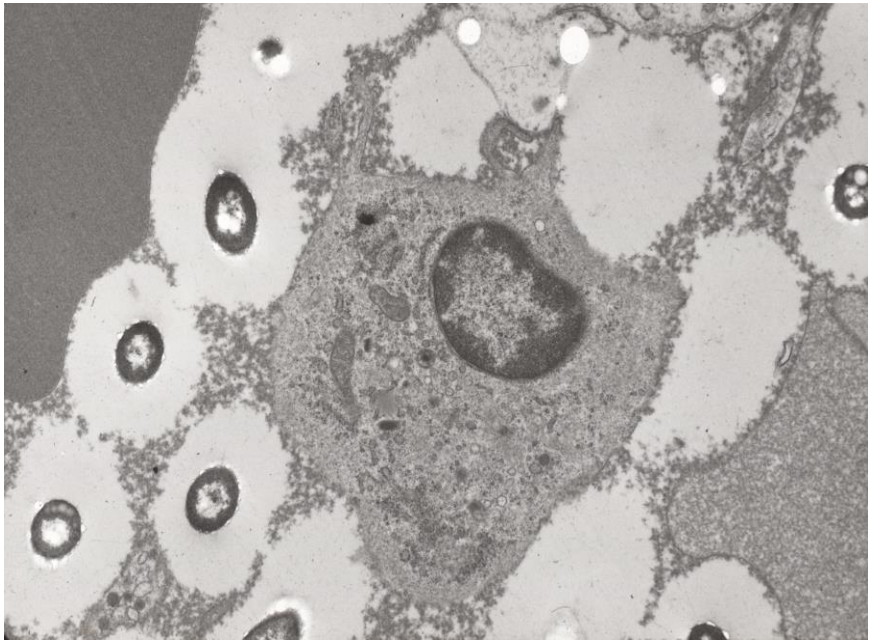
- **pX01 plasmid**
- **AB model**
  - **Binding**
  - **Activating**
- **Protective antigen (PA), edema factor (EF) & lethal factor (LF)**
  - **Make up 50% of proteins in the organism**
- **Individually non-toxic**
  - **PA+LF → lethal activity**
  - **EF+PA → edema**
  - **EF+LF → inactive**
  - **PA+LF+EF → edema & necrosis; lethal**



http://www.rcsb.org/pdb/molecules/pdb28_1.html

Toxins (2)

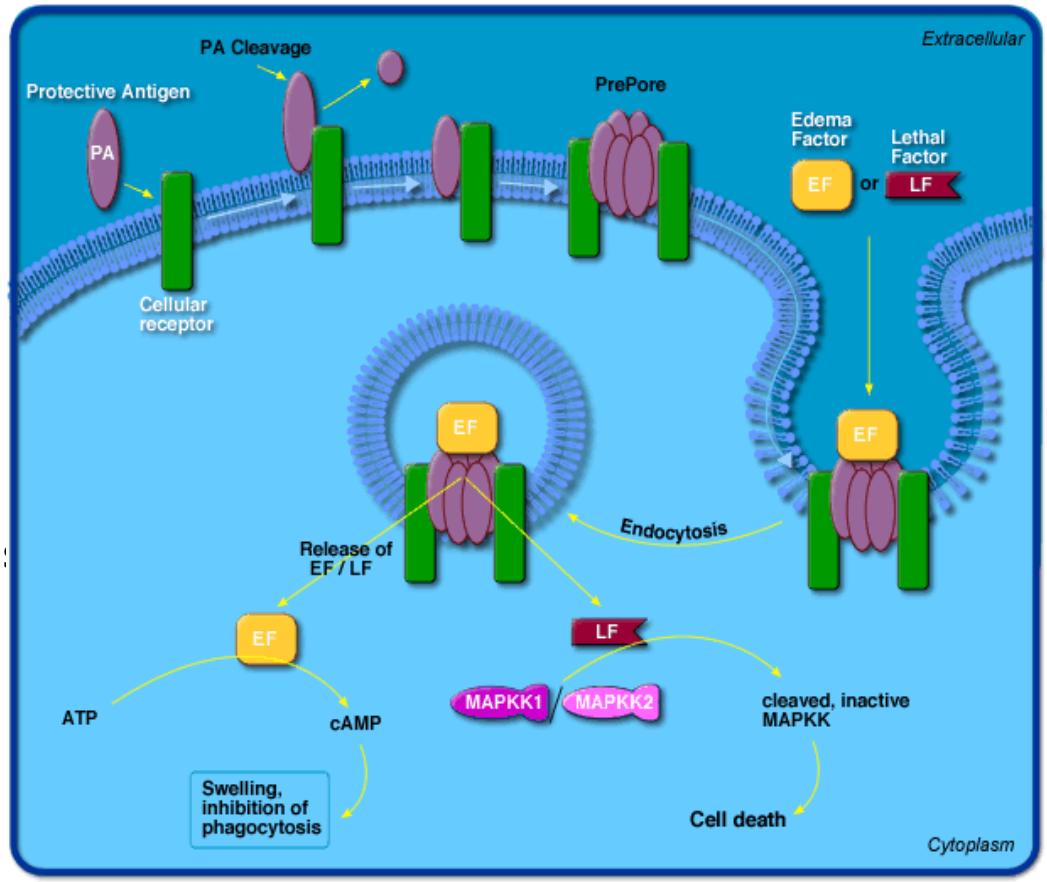
- **Protective antigen (PA, 83kDa)**
  - ***Pag* gene**
  - **Binds to receptor & helps internalize other 2 proteins**
- **Edema factor (EF, 89 kDa)**
  - ***Cya* gene**
  - **Adenylate cyclase**
  - **Affects all cells**
- **Lethal factor (LF, 87 kDa)**
  - ***Lef* gene**
  - **More important virulence factor**
  - **Metalloprotease**
  - **Cleaves mitogen activated protein kinase kinsase (MAPKK)**
  - **Affects only macrophages**



http://www.ericse.org/anthrax/anthraxmicrogra

Mechanism of Infection

- Anthrax spores enter body
- Germinate & multiple in lymph nodes
- PA, EF, LF excreted from bacteria
- PA binds to TEM8.
- PA nicked by protease furin
  - 20-kDa segment off leaving 63-kDa peptide
  - Heptamer forms
- EF and/or LF binds
- Complex internalized by endocytosis
- Acidification of endosome
- LF or EF crosses into cytosol via PA mediated ion-conductive channels
- LF cleaves MAPKK 1 & 2
- EF stimulates cAMP



http://kugi.kribb.re.kr/KUGI/Pathways/BioCarta/anth

Outcome

- Do not understand exactly how symptoms occur
- EF converts ATP to cAMP
  - Increases cAMP levels over 1,000 fold
  - Impairs neutrophil function
  - Alters water homeostasis
  - Edema
- LF cleaves MAPKK at its N terminus
  - Disrupts pathways involved in cell growth & maturation
  - Increased synthesis of tumor necrosis factor-α & interleukin-1β
  - Macrophage lysis
  - More cells infected with bacteria & toxin
  - Septic shock & death
- Death probably results from high levels of bacteria secreting LF toxins in blood
  - At death, blood contains as many as 109 bacilli/ml (depending on the species)

Regulators

- **Bicarbonate or CO_2 stimulates capsule and PA formation**
- **LF requires zinc ions**
- **EF requires calmodulin, a major intracellular calcium receptor**
- **Transcriptional regulator *AcpA* on pX02 controls expression of capsule**
- ***atxA* on pX01 is a positive regulator necessary for transcription of all 3 toxin genes**

Clinical Information

- **Infection**
- **Symptoms (1st and 2nd phase)**
- **Three forms of Anthrax infection and their Pathology**
- **Diagnosis**

Infection of Anthrax

- **The estimated number of naturally occurring human cases of anthrax in the world is 20,000 to 100,000 per year.**
- **Humans are infected through contact with infected animals and their products because of human intervention.**
- **Anthrax spores contaminate the ground when an affected animal dies and can live in the soil for many years.**
- **Anthrax can also be spread by eating undercooked meat from infected animals.**
- **Anthrax is NOT transmitted from person to person.**
- **Humans can be exposed but not be infected.**

What are the symptoms for anthrax?

- **There are two phases of symptom.**
- **1) Early phase - Many symptoms can occur within 7 days of infection**
- **2) 2nd phase - Will hit hard, and usually occurs within 2 or 3 days after the early phase.**

Three clinical forms of Anthrax

- **3 types of anthrax infection occur in humans:**

  1) Cutaneous

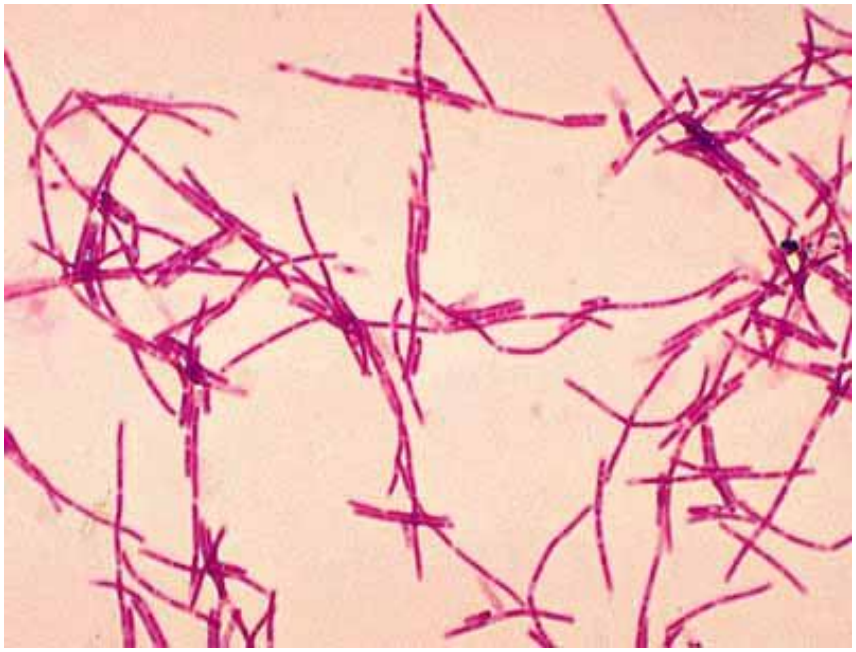
  2) Inhalation

  3) GI

How is anthrax diagnosed?

- **Gram stain**
- **Culture of *B. anthracis* from the blood, skin lesions, vesicular fluid, or respiratory secretions**
- **X-ray and Computed Tomography (CT) scan**
- **Rapid detection methods**
  - PCR for detection of nucleic acid
  - ELISA assay for antigen detection
  - Other immunohistochemical and immunoflourescence examinations
  - These are available only at certain labs

Gram Stain Analysis



Bacillus anthracis in Gram stain

- Useful for cutaneous and inhalation anthrax.
- A blood sample or skin lesion is taken from the patient and cultured for 6 to 24 hours.
- Gram stain takes about 10 to 15 minutes.
- Identify whether the bacteria come from the anthrax category.

PCR Assay

- **PCR is a target amplification method of nucleic acid based B. anthracis detection.**
- **Used for the detection of anthrax toxin genes.**
  ex) rpoB gene - used as a specific chromosomal marker for RT-PCR detection.
- **The rpoB gene was sequenced from 36 Bacillus strains**
- **The assay was specific for 144 Bacillus anthracis strains from different geographical locations.**
- **Provided 100% sensitivity and specificity**

EDEMA FACTOR
(Edema Factor + Protective Ag = Edema toxin)

Calmodulin dependent adenyl cyclase

Increased cellular cAMP ⟶ Edema ⟶ Impaired Neutrophil function

Depletes ATP from Macrophages

LETHAL FACTOR
(Lethal Factor + Protective Ag = Lethal toxin)

Zinc metallo proteases that inactivates protein kinases

Stimulates Macrophages – TNF alpha and IL – 1 beta – Shock & Death

Death due to oxygen depletion, secondary shock, increased vascular permeability, respiratory failure and cardiac failure.

Sudden and unexpected.

Virulence of Anthrax bacillus is due to presence of two plasmids

px01 – Toxin encoding plasmid
- 110 megadalton
- temperature-sensitive **plasmid**

px02 - Capsule encoding plasmid (3 genes - cap A, cap B, cap C)
- 60 megadalton plasmid
- synthesis of poly glutamic acid capsule

Both plasmids are required for virulence

- loss of either - attenuation
- genes expressed only in vegetative state

Pasteur strain - Encapsulated

Sterne strain – Non encapsulated

LABORATORY DIAGNOSIS

Few points to remember

- **Anthrax is not highly contagious**
- **Cutaneous anthrax is not lethal and is readily treated with common antibiotics**
- **ID for human pulmonary / intestinal infection is > 10,000 spores**

SPECIMEN TO COLLECT (HUMAN ANTHRAX)

Disposable gloves, masks, overalls, boots, head gear and dust mask

Disposable items – Autoclave and incinerate

Cutaneous anthrax: Vesicular exudate – swabs and capillary tube aspirate

Intestinal anthrax: - Stool sample - isolate – guinea pig inoculation
- Blood(venipuncture) smear examination for bacilli
- Peritoneal fluid for culture
- Paired sera for Ab

Pulmonary anthrax: If mild disease (No sample)
Severely ill – Blood , sputum, serum samples for Ab

SAMPLES FROM ANIMAL

Sudden death of animal in areas where anthrax was reported earlier

Carcasses 1 or 2 day old
Aspirate blood - MacFadyean stain for bacilli
Direct demonstration by IFA
Direct plating on blood agar

Putrefying carcasses
Blood, tissue and hide
Culture on selective medium
Soil sample from the areas where the carcass as lying

Serological assay: **Ascoli's test**

ELISA: based on anthrax toxin (PA, LF and EF) for routine confirmation and vaccine response)
Molecular techniques (Only in the referral laboratories):
- RFLP
- PCR Fingerprinting
Animal Inoculation: Guinea pig and mice inoculation

Culture is confirmed by gamma phage lysis (PlyG lysin enzyme- g phage)

Table 44. Main diseases and hosts of the *Bacillus* species.

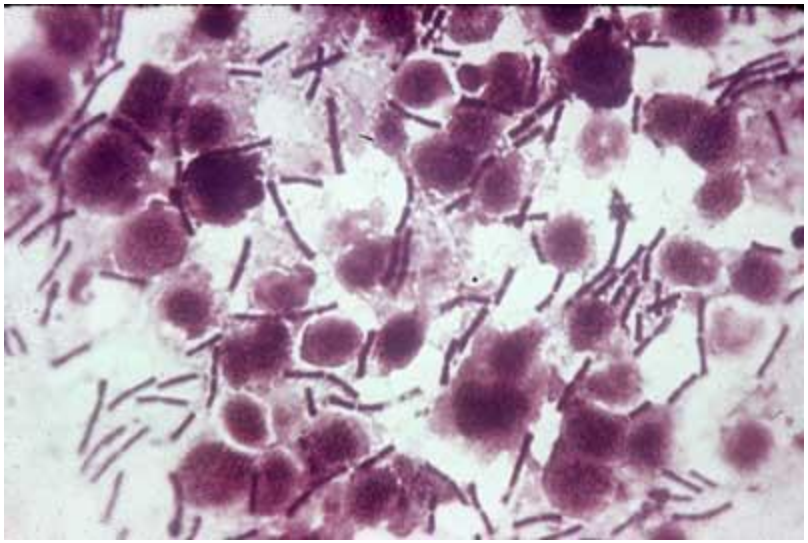
Bacillus species	Host(s)	Disease
B. anthracis	Cattle and sheep	Septicaemic form of anthrax. Usually sudden death
	Pigs	Subacute anthrax with oedematous swelling in pharyngeal tissues and regional lymphadenitis or intestinal form with a higher mortality
	Horses	Oral route: septicaemia with colic and enteritis. Wound infections: localised oedema and lymphadenitis
	Carnivores (including mink)	Comparatively resistant. Disease pattern similar to that in pigs. A massive dose from eating anthrax-infected carcasses can lead to septicaemia
	Humans	Skin form: 'malignant pustule'. Pulmonary ('wool-sorters' disease') and intestinal forms are often fatal
B. cereus	Humans	Food poisoning
	Cattle	Rare cases of mastitis
B. licheniformis	Cattle and sheep	Reported as a cause of abortion
'B. piliformis' (taxonomy uncertain)	Laboratory mice, foals and other animals	Tyzzer's disease. An acute fatal infection causing hepatitis, enteritis and colitis

Laboratory Diagnosis: *Bacillus anthracis*

- Microscopic morphology
  - Gram stain: large, square-ended gram-positive/gram- variable rods; may appear end-to-end giving a "bamboo appearance”
- Colonial morphology
  - Nonhemolytic on 5% blood agar; raised, large, grayish-white, irregular, fingerlike edges described as “Medusa head” or “beaten egg whites” (colony stands upright when lifted with loop)

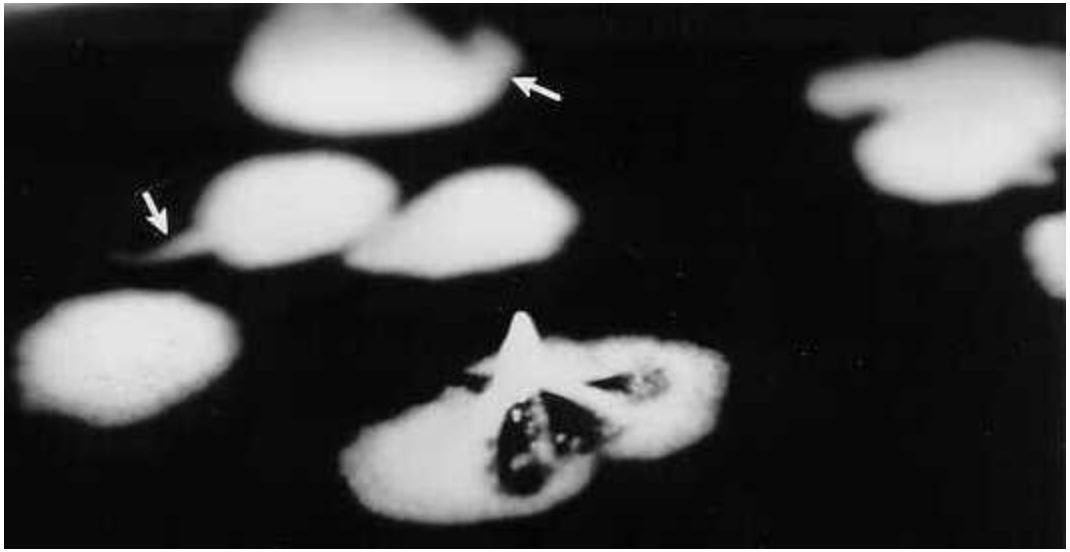
Laboratory Diagnosis: *Bacillus anthracis*

- *B. anthracis* in a gram stain from a cutaneous lesion



Laboratory Diagnosis:
Bacillus anthracis

- *B. anthracis* colonies showing finger-like edges and “beaten egg whites” consistency



IMMUNITY TO ANTHRAX

Resistance against anthrax vary from species to species

- Human are partially immune to anthrax

Resistance can be of two types

- Resistance to the establishment of infection but sensitive to toxin
- Resistance to toxin but susceptible to infection

Animals surviving naturally acquired anthrax are immune to reinfection

Protective antibodies against the anthrax toxin and against the capsule

Resistance to Anthrax vary from species to species

Animal model	**Infectious dose**	**Toxic dose causing death**	**Bacteria per ml blood at time death**
Mouse	5 cells	1000 units/kg	10^7
Monkey	3000 cells	2500 unit/kg	10^7
Rat	10^6 cells	15 units/kg	10^5