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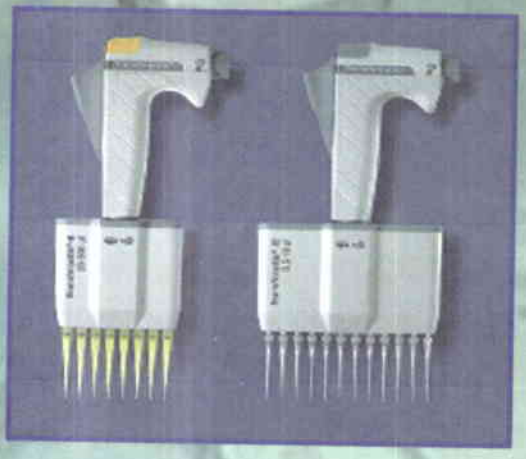
المنظمة العربية للتنمية الزراعية

الدورة التدريبية الإقليمية حول التطبيقات العملية للتقنيات المتقدمة لتشخيص مرض الإجهاض المعدي

2006/6/15 - 11

القاهرة - جمهورية مصر العربية



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المدير العام للمنظمة العربية للتنمية الزراعية

في الجلسة الإفتتاحية للدورة التدريبية الثالثة حول

التطبيقات العملية للتقنيات المتقدمة في مجال تشخيص وإستقصاء

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بسم الله الرحمن الرحيم

معالي الأخ المهندس أمين أباظة وزير الزراعة واستصلاح الأراضي

الإخوة المتدربون

السيدات والسادة الحضور

السلام عليكم ورحمة الله وبركاته

إنه لمن دواعي سروري وسعادتي أن أرحب بكم بالأصالة عن نفسي ونيابة عن أسرة المنظمة العربية للتنمية الزراعية في حفل إفتتاح الدورة التدريبية الإقليمية الثالثة حول التطبيقات العملية للتقنيات المتقدمة لتشخيص واستقصاء مرض الإجهاض المعدي التي تنفذها المنظمة العربية بالتعاون مع وزارة الزراعة واستصلاح الأراضي بجمهورية مصر العربية.

ويسرني بداية أن أتقدم بالشكر وعظيم التقدير إلى جمهورية مصر العربية رئيساً وحكومةً وشعباً لاحتضانها واستضافتها لهذه الدورة ودعمها المتواصل للعمل العربي المشترك ومؤسساته.

وأود أن أتقدم بعميق الشكر والتقدير لمعالي الأخ المهندس أمين أباظة على رعايته الكريمة لهذه الدورة.

كما أتقدم بعاطر الثناء وجزيل الشكر للإخوة القائمين على تنفيذ هذه الدورة من الهيئة العامة للخدمات البيطرية ومعهد بحوث صحة الحيوان ، كما يسعدني أن أرحب بالإخوة المشاركين من الدول العربية متمنياً لهم طيب الإقامة في بلدهم الثاني مصر والتوفيق والنجاح في المجال العملي.

معالي الوزير

السيدات والسادة

يمتلك وطننا العربي ثروة حيوانية هائلة ، وعلى الرغم من اتساع هذه الثروة إلا أن طاقتها الإنتاجية الحالية تعد ضعيفة ولا تلبي الإحتياجات الحالية للوطن العربي . تعدّ الصحة الحيوانية إحدى

دعائم ثلاثة ترتكز عليها تنمية الثروة الحيوانية وهي تحسين الصفات الوراثية والتغذية والرعاية الصحية. هذا ورغم الجهود الكبيرة التي تقوم بها الدول العربية في مجال الرعاية الصحية إلا أن ثروتها الحيوانية لا زالت تعاني من ظروف صحية قاسية تتمثل في تفشي أمراض عديدة مثل الحمى القلاعية ، الإجهاض المعدي ، طاعون المجترات الصغيرة إضافة إلى تفشي آفات مثل القراد والدودة الحلزونية وغيرها وأخيراً أنفلونزا الطيور.

يعتبر مرض الإجهاض المعدي (البروسيللوزس) من أكثر الأمراض شيوعاً وأهمها من الناحية الاقتصادية في الوطن العربي إضافة إلى تأثيره الكبير على الصحة العامة . لقد أدت الطفرة العلمية والتكنولوجية العالية في السنوات القليلة الماضية إلى تطور سريع وواسع في وسائل التشخيص المعملي والأجهزة المستخدمة في هذا المجال واستنباط كواشف عالية النقاء ذات خصائص ثابتة مما أتاح تشخيص أمراض بفاعلية متناهية وتحديد مسببات أمراض خطيرة وتحديد سلالاتها وأنماطها الحيوية بدرجة من الدقة لم تكن ممكنة من قبل . رغم أهمية وفاعلية مثل هذه التقنيات إلاَّ أن إرتفاع أسعار الأجهزة المستخدمة واحتياجها إلى تدريب فني ومران وخبرة علمية عالية جعلا استخدامها في كثير من بلدان الوطن العربي أمراً قاصراً ومحدوداً نسبياً حتى الآن.

وفي إطار هذا المفهوم ، ومواصلة لجهودها في تطوير قطاع الثروة الحيوانية بالدول العربية ، تقدمت المنظمة العربية للتنمية الزراعية بمشروعها القومي للدعم الفني للبرامج الوطنية لمكافحة مرض الإجهاض المعدي ، والذي ساهم مشكوراً في تمويله الصندوق العربي للإنماء الإقتصادي والإجتماعي ، وذلك ضمن برنامج شامل لمكافحة الأوبئة الحيوانية تقوم المنظمة العربية بتنفيذه. يشمل هذا البرنامج أمراضاً وآفات أخرى ذات أهمية كبيرة في الوطن العربي منها الحمى القلاعية ، أنفلونزا الطيور ، الحمى المدارية ، حمى الوادي المتصدع وذبابة الدودة الحلزونية وتعتبر الدورة التدريبية الحالية ، والتي نحن بصددها اليوم حول التطبيقات العملية للتقنيات المتقدمة لتشخيص واستقصاء مرض الإجهاض المعدي، إحدى أنشطة هذا البرنامج الشامل والتي نحن على ثقة كبيرة بنجاحها .

في الختام أكرر الشكر والتقدير لجمهورية مصر العربية على استضافتها لهذه الدورة وأتمنى للإخوة المشاركين كل التوفيق والنجاح.

والسلام عليكم ورحمة الله وبركاته.

المحتويـــــات

المحتويات

Diagnosis of Brucellosis

Diagnosis of Brucellosis

Dr. M. Nabil

A- Field diagnosis

1. History and clinical signs:

The only frequent clinical sign is abortion (on the farm level). Brucellosis is not the only cause of late abortion.

2. P. M. examination:

There is no pathognomonic lesion for brucellosis. Brucellosis is not the only cause of granuloma formation.

3. Allergic skin test:

It is a herd rather than an individual test especially useful for small ruminants. It has a high practical in herds/ flocks where animal ear tagging is not a common practice. It may give false positive results in cattle & buffaloes.

B- Laboratory diagnosis

1. Bacteriological examination (direct):

Direct detection of the causative agent of brucellosis is the only definitive way of diagnosis that has a positive value. When positive, it is 100% reliable, but if negative, it does not exclude Brucella infection. Bacteriological examination is unfortunately time-, money-, effort- & proficiency- demanding. It has a great epidemiological value.

2. Serological examination (indirect):

Serological tests used in the diagnosis of animal brucellosis can be classified, depending on the antigens used, as conventional tests (i.e., those using suspensions of whole cells as antigens) and tests using antigenic extracts. Conventional tests, RBT and CFT in particular, are widely used for cattle and sheep brucellosis **[4]** but gel precipitation with selected *Br. abortus* and *Br. melitensis* polysaccharides **[3, 15, 69, 24, 25,]**; and several ELISAs with S-LPS-rich extracts [4, 69], have also been proposed. All the above tests have in common that they detect mostly antibodies to antigenic determinants present in the O chain of the S-LPS.

Serotests for brucellosis as recommended by the OIE

A-Presumptive or screening tests:

1. Buffered acidified plate antigen test (BAPA).

2. Rose-Bengal plate test (RBPT).
3. Milk ring test (MRT) or Abortus Bang ring test (ABR).
4. Indirect ELISA (id ELISA).

B-Supplemental or confirmatory tests:

1. Standard tube agglutination test (SAT).
2. Mercaptoethanol tube agglutination test (MET).
3. Rivanol-precipitation plate agglutination test (Riv. T).
4. Complement fixation test (CFT).
5. Competitive ELISA (c ELISA).

Serum Agglutination Test (SAT)

Serum Agglutination Test (SAT)

By Dr.Essam Eldin M. Bayoumi

It is a quantitative test used to detect of immunoglobulins classes. The test was carried out according to the method used by the Central Veterinary Laboratory, Weybridge, England and described by (Alton et al., 1988).

- Antibody selectivity: SAT

Antibody	IgM	IgG_1	IgG_2	IgA
Detection	++++	±	++++	++

Test procedure:

* A raw of seven Wasserman tubes were used per sample.

* An amount of 0.08 ml of phenol saline was placed in first tube and 0.5ml was placed in each of the remaining tube.

* To the first tube, 0.2 ml of the serum to be tested was addedand thoroughly mixed, then 0.5 of the mixture was transferred to the seconed tube, from which, (after mixing), 0.5 ml of the diluted serum dilution was transferred to the next tube and so on then 0.5 ml of diluted serum from the last tube was discarded. This results in two fold dilutions of serum (1/5,1/10,1/20, and so on)

* By using an automatic pipette, 0.5 ml of diluted standered antigen was added to each tube.(standerd antigen was diluted 1ml: 4ml using phenol saline).

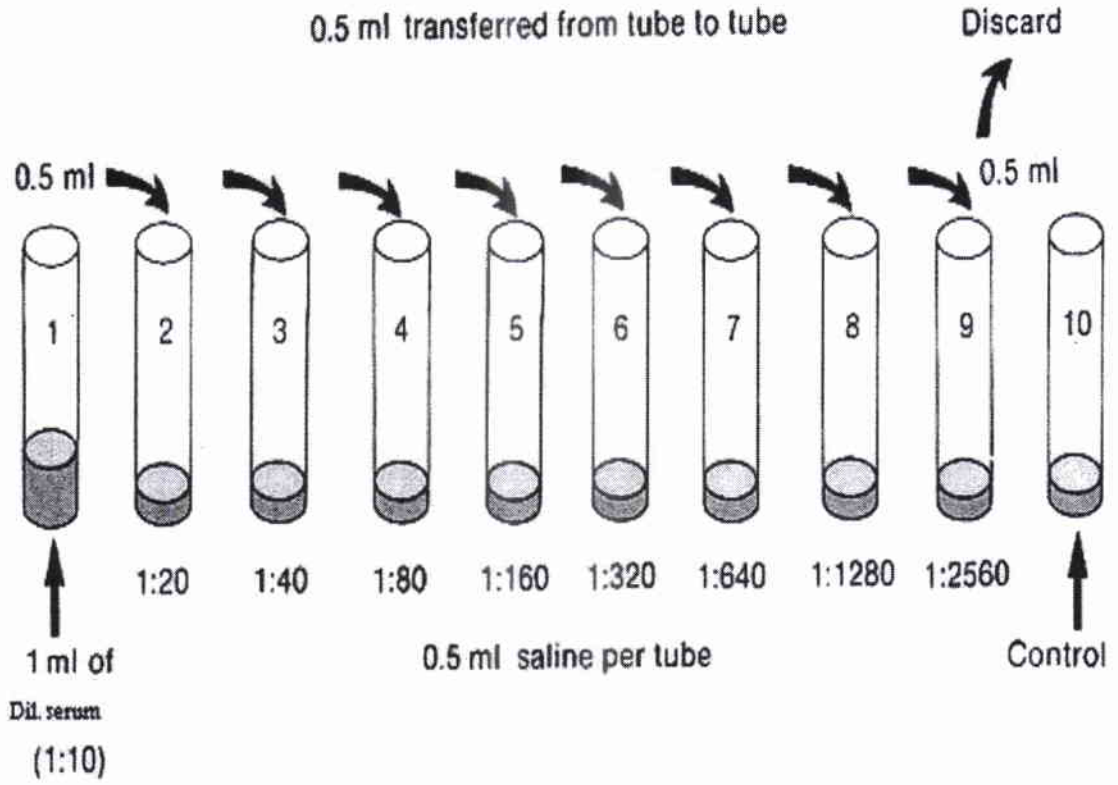
* The contents of the tube was thoroughly mixed by shaking the rackes. This gives final dilutions 1/10, 1/20, 1/40.....etc.

* The tubes were incubated at 37°c overnight befor recording the results and they were examined without shaking against a black background with the light coming from above and behind the tubes .

* The results of agglutination were determined by degree of clumping and clearence without shaking the tubes

* The end titre was the highest serum dilution that gave 50%or more(≥ ++) of agglutination.

* For testing sera of sheep and goats it is recommended that a 5% sodium chloridev soilutioin containing 0.55 phenol be used both for making the serum dilution and for diluting the antigen concentrate.



Agglutination control standerds for reading the SAT

degree of agglutination					
consituent	++++	+++	++	+	-
phenol saline(ml)	1	0.75	0.5	0.25	0
Double Dil. Antigen	0	0.25	0.5	0.75	1

Standards for TAT

++++	Complete agglutination with water-clear supernatant
+++	Nearly complete agglutination and 75% clearing
++	Marked agglutination with 50% clearing
+	Slight agglutination and 25% clearing.
-	No clearing (no agglutination)

Conversion of serum agglutination test titre to IU Ab/ml

Due to variation in denisty of antigens, agglutination tests in different countries may result in different titres even for the same positive serum, Adoption of an international unitage system was done as recommended by the joint FAO/WHO expert committee on brucellosis(1953), and conversion of serum agglutination titres into IU/ml as shown in Table(3)

Using an ampoule of the 2nd (ISAbs), that contains 1000IU/ml and an antigen that gives a titre 1:500, thus a serum givinga titre of 1/20 contains (1000X 20) /500 = 40 IU/ml

Conversion of brucella TAT titre to international units

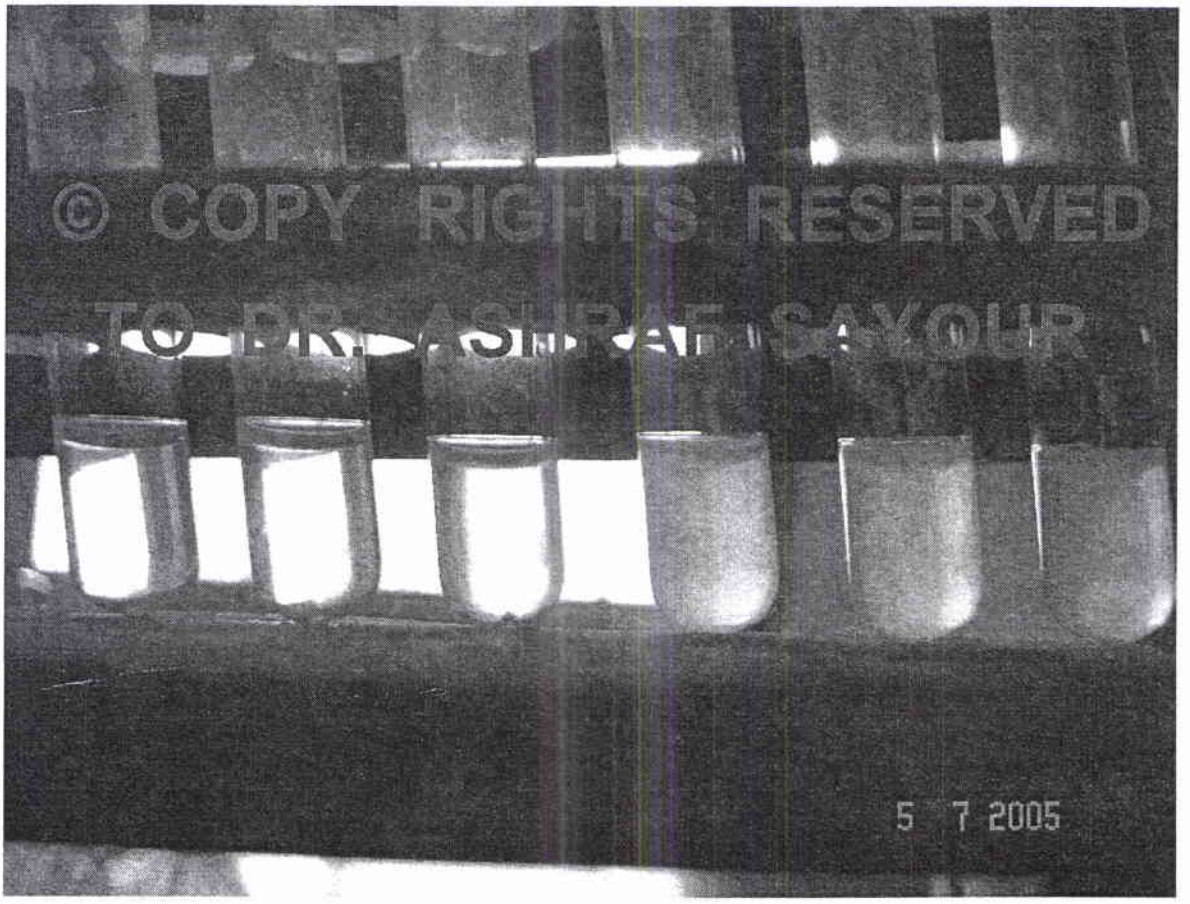
Serum titre	End point	IU.ml $^{-1}$
1/10	+	16.75
	++	20
	+++	23.25
	++++	26.5
1/20	+	33.5
	++	40
	+++	46.5
	++++	53
1/40	+	67
	++	80
	+++	93
	++++	106
1/80	+	134
	++	160
	+++	136
	++++	212
1/160	+	268
	++	320
	+++	372
	++++	424
1/320	+	536
	++	640
	+++	744
	++++	848
1/640	+	1072
	++	1280
	+++	1488
	++++	1696
1/1280	+	2144
	++	2560
	+++	2976
	++++	3392
1/2560	+	4288
	++	5120
	+++	5952
	++++	6784
1/5120	+	5876
	++	10240
	+++	11904
	++++	13568

Int

erpretation of the results of the TAT:

A titre corresponding to 80IU or more Ab/ml was considered positive i.e. titre 1/40 or more.

A titre cocorresponding to 40 IU or less was considered suspicious i.e. titre 1/20 or less.



Mercapto-Ethanol test

This test is an agglutination test carried out in the presence of 2-mercapto-ethanol which inactivate IgM molecules present in the serum being tested. A 0.1 mol/litre mercapto-ethanol solution in normal saline is made as follows.

Sodium Chloride	8.5 gm
2 Mercapto-ethanol	7.14 ml
Distilled water	to 1 litre

The serum dilutions are made in mercapto-Ethanol saline and the antigen concentrate is diluted in normal saline without phenol otherwise the technique is the same as for the traditional test described before.

Interpretation of the results.

Interpretation of the results of mercapto ethanol test should be interpreted alongside with the titers obtained in the ordinary test. Ordinary test indicates the total amount of agglutinins present in the test serum whereas mercapto-ethanol measures the amount of IgG and the difference indicates the proportion of IgM present in the tested serum.

Table 1: Classification of genus *Brucella* into species by phage susceptibility

Brucella species	Lysis by phage groups at routine test dilution (RTD)								
	Group 1	Group 2	Group 3	Group 4	Group 5			Group 6	Group 7
	Tbilisi (Tb)	Firenze (Fi)	Weybridge (Wb)	Berkley (Bk_2)	Rough (R)	R/ ovis (R/O)	R/ canis (R/C)	Izatnagar (Iz_1)	Nepean (Np)
Br. abortus	+	+	+	+	-	±	-	+	+
Br. melitensis	-	-	-	+	-	-	-	+/ ±	-
Br. suis	-	+	±	+	-	±	-	+/ ±	-
Br. neotomae	±	+	+	+	-	-	-	+	-
Br. ovis	-	-	-	-	-	+	+	-	-
Br. canis	-	-	-	-	-	-	-	-	-

\+ **Complete lysis**

± **Partial lysis**

Bacteria Incriminated in Cattle Abortion

Bacteria Incriminated in Cattle Abortion

Dr. Emad M. Riyad

- Many bacteria incremented as a causative agent of abortion other than brucella
- Some microflora of genitalia play a detectable role for inducing abortion in cattle including Salmonella infection

The causative agents are

- Salmonella abortus bovis
- Salmonella dublin
    - It induces abortion at late stage of pregnancy may accompanied by retained placenta.
    - Usually the abortion has no effect on the future fertility of cows unless laceration or acute endometritis takes place.

Types of samples

Listeria

- Gram positive rods facultative anaerobes
- Growth enhanced by 10% CO_2...
- Grow on nutrient and blood agar in a temp. range 43- 45°c within a pH range of 5.6-9.6.
- Incubation at 37°C (24-48 hr).
- *L. monocytogenes* can be excreted in bovine milk.

Samples

- Placenta
- Foetal abomasal content and or uterine discharge.
- Serum for ELISA .

2. Incubate the plate for 48 h at 42-43 °C in a microaerophilic atmosphere obtained by one of the methods described above.

Identification :

Colonial appearance

- Microscopical examination
- Biochemical reactions
- Small beta haemolytic colonies at 24 hs on sheep blood agar .
- Gram +ve rods
- Catalase positive
- Asculin hydrolysis +ve
- On semisoli agar (subsurface umbrella shape)
- CAMP test +ve (S. aureus)
- Antoni test (L. monocytogenes causes purulent keratoconjunctoivitis within 24-36 hs.)
- Pathogenicity in mice :

  Die within 5 days.

CULTURAL CHARACTERISTICS, METABOLISM AND GROWTH REQUIREMENTS:

- Campylobacters are strictly micro-aerophilic and do not grow in air, yet oxygen (5-10%) is normally required for growth.
- The key to successful cultivation of Campylobacters is to add supplements that scavenge these offending compounds. A simple and widely used supplement consists of ferrous sulphate, sodium metabisulphite and sodium pyruvate.

Culture on selective media

1. Fresh samples plated out a plate of one of the recommended selective media, Older specimens may also be plated directly selective media ,as 'long as they have been held at 4°C. for <24h. Enrichment in selective enrichment broth.
2. Incubate the plate for 48 h at 42-43 °C in a microaerophilic atmosphere

obtained by one of the methods described above.

Microscpical examination

- Added to 1 cm thioglycollate broth then examined under the phase contrast microscope using 400 X magnification for defection of the characteristics motility and morphology of Campylobacter organism.

Diagnosis of Campylobacteriosis:

- The genus Campylobacter consists of a well-defined group of bacteria. They are slender, spirally curved rods, 0.5 to 5 μm wide and 0.5 to 5 pm long.
- Campylobacters may be comma, S, or gull wing in shape and may occur in short or occasionally long chains. Cells may become spherical or coccoid, especially in old cultures. The cells are nonsporeforming.

Microscopical examination

- Added to 1 cm thioglycollate broth then examined under the phase contrast microscope using 400 X magnification for defection of the characteristics motility and morphology of Campylobacter organism.

Serodioagnosis:

- Enzyme Linked Immuno Sorbent Assay.
- Passive Haemagglutination Test.
- Slide Agglutination Test .
- Serodiagnosis
- Indirect ELISA was carried out for detection of specific immunoglobulin against infectious agents

Non competitive method

- Indirect method
- This is the system which can be used for the detection and measurement of antibodies
- It is carried out as follows
- 1- antigen attached to the solid phase which is then washed.
- Diluted test serum is added incubate, wash.

- An enzyme labeled (anti species)is added incubate, wash
- Enzyme substrate is added colour developed
- Read the plate visually and at spectrophotometer.
- Amount of antibody in test serum is related to the colour change

Enzyme-linked immunosorbent assay (ELISA)

Principle :

It is an enzyme-labelled antiglobulin test in which the antigen is adsorbed on polystyrene microtitre plate. The sample under test is added, any specific antibody present binds to the antigen, then the other serum constituents are removed by washing. The bound antibody is demonstrated by adding an antiglobulin serum conjugated with an enzyme capable of generating a colour change when a suitable substrate is added.

Test procedure:

i) LPS antigen is diluted in coating buffer to a concentration approximately 1μg/ml, and dispensed to all microwells in 100μl volumes. The microplates are then incubated at 4°C overnight.

ii) Test sera are diluted to 1/200 to appropriate wells on the plate. The plates are re-covered or sealed and placed on an orbital shaker and incubated at 37°C for ½-1 hour with continuous shaking.

iii) Replicate positive and negative working standards treated identically to the test sera and a blank well, to which no serum is added must be included on every plate.

Wash the plate.

iv) The enzyme conjugate is diluted in diluent buffer and applied to all wells in 100μl volumes. The plates are covered or sealed and placed on an orbital plate shaker and incubated at 37°C for ½-1 hour with continuous shaking.

Wash the plate.

v) Fresh substrate/chromogen solution is prepared by adding 60μl of the 3% hydrogen peroxide stock solution and 300 □l ABTS chromogen stock solution to 12ml of substrate buffer. The substrate-chromogen solution is applied to all wells in 100μl volumes. The plates are incubated at RT for up to 15 minutes.

vi) After 15 minutes incubation, the enzymatic reaction stopping solution is applied to all wells in 100μl volumes and the plate is shaken briefly on the plate shaker to ensure thorough mixing. All wells now contain a total volume of 200μl.

Reading of results and calculation:

vi) The optical density (OD) is read with a microplate spectrophotometer using a 405 or 414nm interference filter. The OD of the blank well should be subtracted from the OD of all other wells.

vii) The data may be expressed in a number of different ways, but it is recommended that OD of each test serum be expressed as per cent positivity (PP). This is calculated for each plate by expressing the OD of the test serum as a percentage of the mean OD of a working standard serum adjusted to be equivalent to the OIE International Standard Strong Positive ELISA Serum.

All indirect ELISAs should be standardized such that the strong positive standard gives an OD of between 0.5 and 1.0 OD units. The weak positive standard must always give a positive reaction of between 20-30% PP whilst a ¼ dilution of this must always be negative. The negative standard must always give a reaction of less than 10% PP.

Antibody isotype selectivity

The different classes of immunoglobulins can be identified by using antiglobulin conjugates prepared against particular isotypes. Unlike conventional tests, ELISA is effective in detecting nearly all immunoglobulin classes and subclasses irrespective to their biological activity.

Antigens used

Many antigens have been tried including whole cells, sonicated antigen, autoclaved antigen and poly B. The most common antigens include LPS and outer membrane proteins.

Uses

- Early detection of antibodies for presumptive diagnosis of brucellosis or for confirmatory diagnosis when competitive ELISA is used.
- Being flexible, the test is suitable for research purposes.

Advantages

- Fexibility in terms of ability to use different techniques, reagents and antigens which determines sensitivity and specificity.
- Being primary binding assay, it has improved sensitivity (ELISA) or specificity (cELISA) when compared to the classical secondary binding assays.
- Major focus for the development of computer controlled automation.

Disadvantages

- Semi-quantitative when standardized carefully, but not an accurately quantitative assay.
- Expensive if used as a screening tool or if monoclonal reagents are included. Other tests as the BAPA are economical, rapid and equally sensitive.
- Enzyme activity may sometimes be affected by plasma constituents.
- Differential adsorptive characteristics of polystyrene.
- Nonspecific reactions especially when monoclonals are not employed.

Introduction to the Basic Principle of Molecular Biology

Introduction to the Basic Principle Of Molecular Biology

Prof. Dr.G.H. Salem

Chief Researcher and Head of
Department of Biotechnology ,Animal Health Institute

Introduction

The heritable property of an organism is controlled by a factor, called a gene. The discovery of Mendel's laws in 1900 makes the birth of genetics .

There is no substance so important as DNA . because it carries within its structure the hereditary information that determines the structures of proteins, it is the prime molecule of life. The instructions that direct cells to differentiation of fertilized eggs into the multitude of specialized cells that are necessary for the successful functioning of higher plants and animals.

Nucleic acids are macromolecules of biological importance. All living organisms contain nucleic acids in the form of deoxyribonucleic acid (DNA) and ribonucleic acid (RNA).Some viruses may contain only RNA,while others have only DNA . DNA is the major store of genetic information. This information is transmitted by transcription into RNA molecules, which are utilized in the synthesis of proteins. In fact, the central dogma of the modern biology is :

DNA - RNA - PROTEIN.

In higher cells DNA is localized mainly in the nucleus a

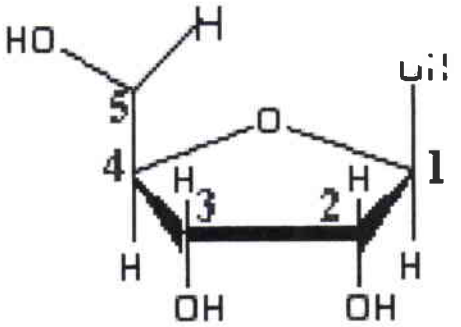
part of the chromosomes. A small amount of DNA is present in the cytoplasm and contained within mitochondria and chloroplasts. RNA is found both in the nucleus, where it is synthesized,and in the cytoplasm, where the synthesis of proteins occurs.

DNA and RNA : structure and reactions role in the cell.

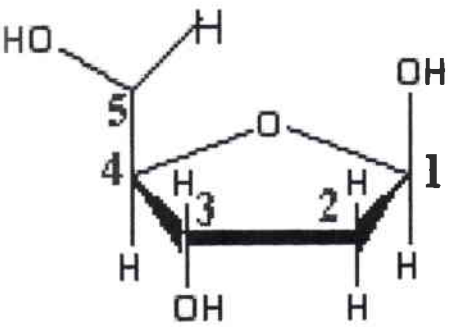
	Deoxyribonucleic Acid	*Ribonucleic Acid*
Localization	**Primarily in nucleus, also in mitochondria and chloroplasts.**	**In cytoplasm. nucleous and chromosomes.**
Pyrimidine bases	**Cytosine , Thymine**	**Cytosine , Uracil**
Purine bases	**Adenine , Guanine**	**Adenine ,Guanine**
Pentose	**Deoxyribose**	**Ribose**
Hydrolizing enzyme	**Deoxyribonuclease(DNase)**	**Ribonuclease(RNase)**
Role in cell	**Genetic information**	**Synthesis of proteins**

Structure of nucleic acids :

Nucleic acids are formed of sugar (pentose) , nitrogen bases (purines and pyrimidines) and phosphoric acid. After a mild hydrolysis the nucleic acids are decomposed into nucleotides.

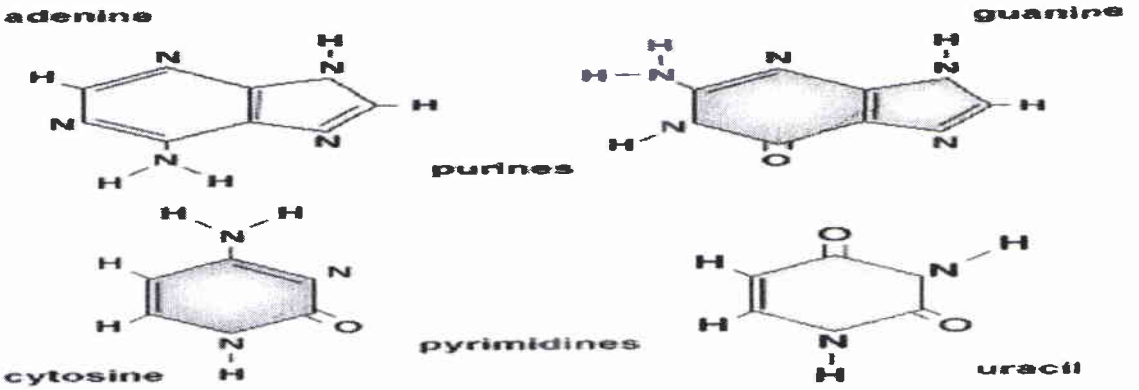


Ribose



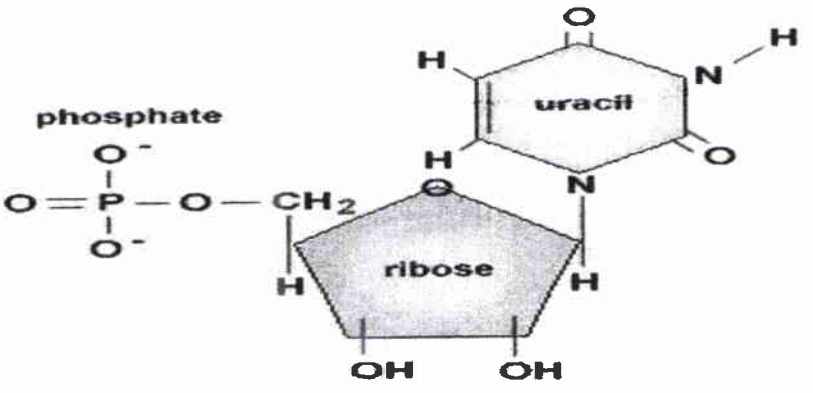
Deoxyribose

Pentose of nucleic acids showed the difference in carbon atom 2'.



Nitrogen bases (purine and pyrimidine)

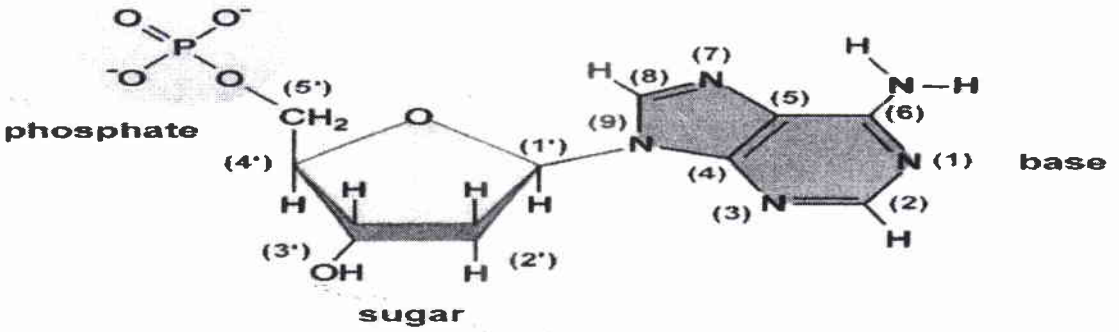
Nucleotides are the monomeric units of the nucleic acid macromolecule.The nucleotide results from the covalent bonding of a phosphate and a heterocyclic base to the pentose. Within the nucleotide, the combination of a base with the pentose constitutes a nucleoside.



bonding of a phosphate and a hetrocyclic base to the pentose

For example, adenine is a purine base; adenosine (adenine + ribose) is the corresponding nucleoside and adenosine monophosphate (AMP), adenosine diphosphate (ADP), and adenosine triphophate (ATP) are nucleotides. In addition to constituting the building blocks of nucleic acids, nucleotides are important because they are used to store and transfer chemical energy.

Nucleic acids are linear polymers in which the nucleotides are linked together by means of phosphate- diester bridges with the pentose moiety. These bonds link the 3' carbone in one nucleotide to the 5' carbon in the pentose of the adjacent nucleotide. The backbone of nucleic acids consists, therefore, of alternating phosphates and pentose. The nitrogenous bases are attached to the sugars of this backbone.



The structure of a nucleotide of DNA

The phosphoric acid uses two of its three acid groups in the 3', 5' diester links. The remaining negative group confers to the polynucleotide its acid properties and enables the molecule to form ionic bonds with basic proteins.In eukaryotic cells,DNA is associated with histones (i.e basic proteins rich in arginine or lysine) forming a nucleoprotein complex called chromatin. This anionic group also causes nucleic acids to be highly basophilic; i.e., they stain readily with basic dyes.

Pentose are of two types: ribose in RNA , and deoxyribose in DNA . The only difference between these two sugars is that the oxygen in the 2' carbon is lacking in deoxyribose.

The bases found in nucleic acids are either pyrimidines or purines. Pyrimidines have a single heterocyclic ring, whereas purines have two fused rings. In DNA the

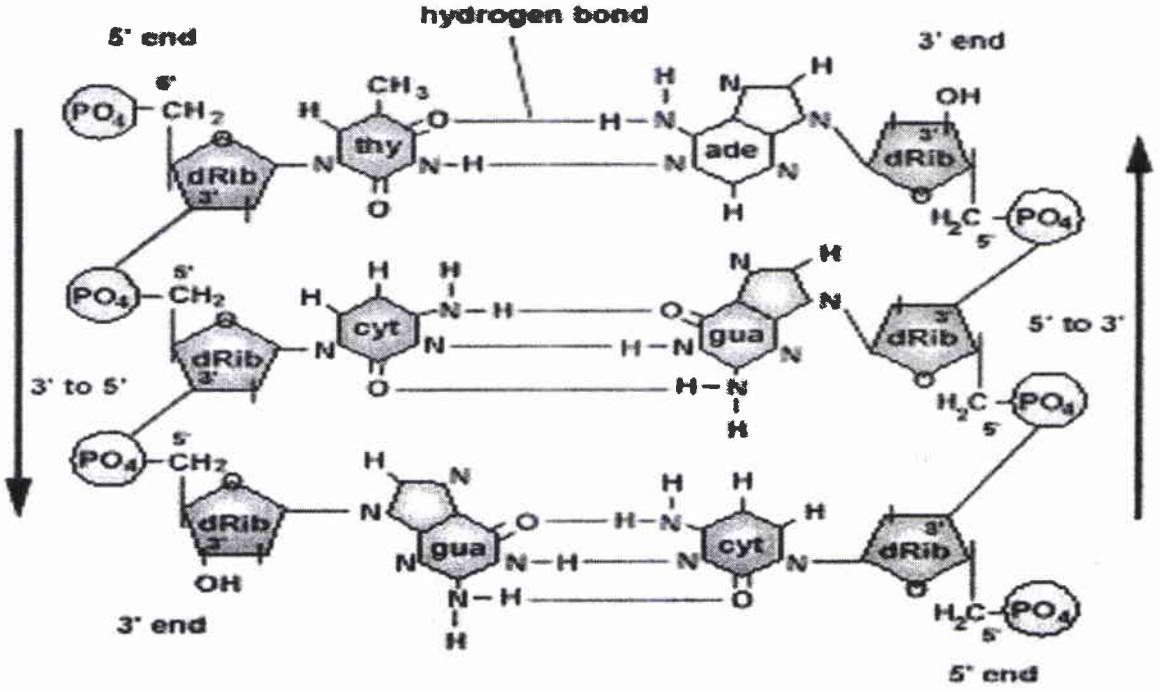
pyrimidines are thymine (T) and cytosine (C) . RNA contains uracil (U) instead of thymine.

Therefore between RNA and DNA there are two main differences: the first one in the pentose moiety (ribose and deoxyribose, respectively)and the second in a pyrimidine base (uracil instead of thyamine). This explains why in cell biology radioactive; thymidine(i.e., the nucleoside) is used to label specifically DNA and radioactive uridine for RNA.

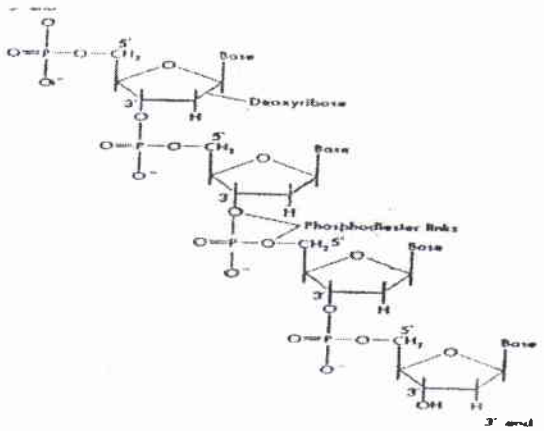
All the genetic information of a living organism is stored in the linear sequence of the four bases. Therefore, a four letter alphabet (A, T, C,G) must code for the primay structure of all proteins (i.e, composed of 20 amino acids) . All the excitement in molecular biology, leading to the unraveling of the genetic code,began when the structure of DNA was understood.

DNA is a Double Helix :

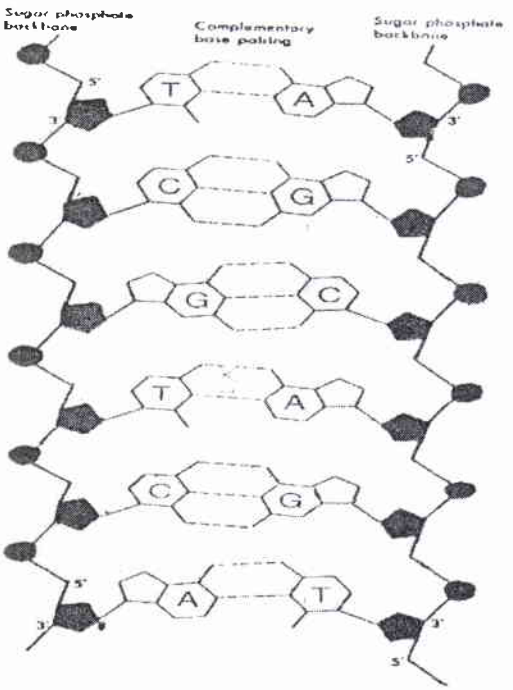
In 1953, based on the x-ray diffraction data of Wilkins and Franklin, Watson and Crick proposed a model for the DNA structure that provided an explanation for the regularities in bases composition and for the biological properties of DNA – particularly its duplication in the cell.



The structure of DNA is composd of two right –handed helical polynucleotide chains that form a double helix around the same central axis. The two strands are antiparallel; i.e., their 3' , 5' phosphodiester links are in opposite directions. Furthermore, the bases are stacked inside the helix in a plane perpendicular to the helical axis.



Regular phosphodiester bonds between sugar and phosphate groups form the backbone of DNA.



he base pairing of two DNA chains.

The base pairing of two DNA chain

The two strands are held together by hydrogen bonds established between the pairs of bases . Since there is a fixed distance between the two sugar moieties in the opposite strands, only certain base pairs can fit into the structure.

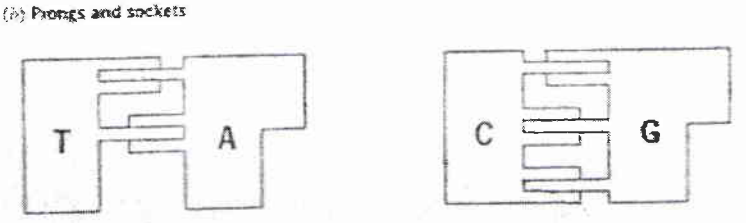
Double helix of DNA and the nitrogen bonds bind nucleotides

The only two pairs that are possible are AT and CG. It is important to note that two hydrogen bonds are formed between A and T , and three are formed between C and G , and that therefore a CG pair is more stable than an AT pair . In addition to hydrogen bonds, hydrophobic interactions established between the stacked bases are important in maintaining the double helical structure.The axial sequence of bases along one polynucleotide chain may vary considerably, but on the other chain the sequence must be complementary.

Because of this property, given an order of bases on one chain, the other chain is exactly complementary as described below :

3' T,T,G,C,T,A,T,A,A,T,C,C,G, 5' first chain

5' A,A,C,G,A,T,A,T,T,A,G,G,C, 3' second chain



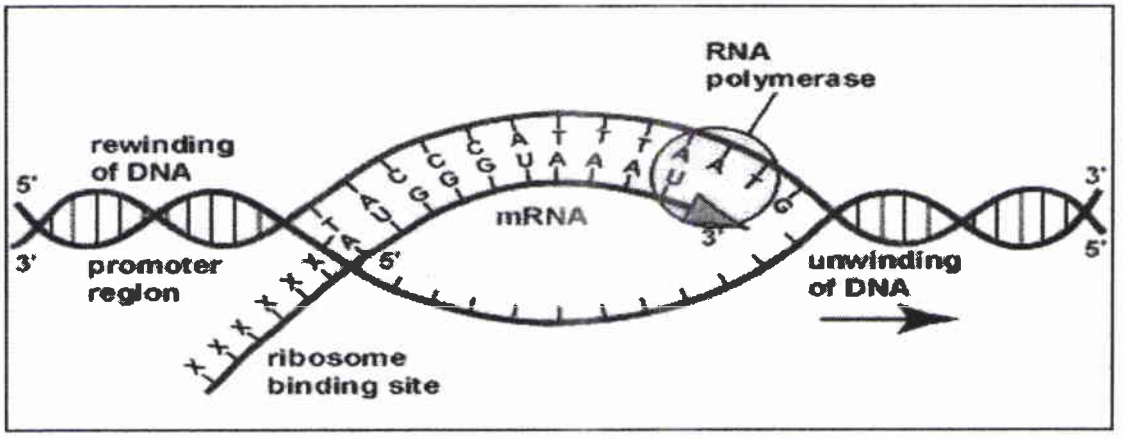
Complementary base pairing ; A prong s-and-sockets analoge for base pairing , the hydrogen atoms in each hydrogen bond are represented as prongs , and the oxygen or nitrogen atoms are depicted as sockets. The attractive forces are weak; consequently , perfect fits are required for base-pairing to occur .

Four bases can produce thousands of different hereditary characters, because DNA molecules are long polymers along which an immense number of combinations may be produced.

During DNA duplication, the two chains dissociated , and each one serve as a template for the synthesis of two complementary chains. In this way two DNA molecules are produced, each having exactly the same molecular constitution.

Gene expression : Transcription

Gene expression is the process of producing a protein from the information stored in DNA This process involves from specific regions of the DNA is made available to the rest of the cell through a process called transcription. In this process an enzyme, RNA polymerase, recognized and binds to a DNA nucleotide sequence near one end of a gene. This recognition and binding allows RNA polymerase to select the proper DNA strand (the strands are complementary, not identical) to use as a source of information.The polymerase then moves into the gene. As RNA polymerase moves , it creates a new chain by linking together RNA nucleotides floating free in the cell. The order of the nucleotides in the new chain is determined by the complementary base-pairing rule. If the first letter the RNA polymerase encounters in the DNA is a (T), the enzyme will add an (A)to the new chain it is making.Likewise, if the next DNA letter is (C), a (G)is added to the new chain.Eventually a stop signal at the end of the gene is reached , RNA polymerase comes off the DNA , and the new chain is released; this new chain is called RNA.



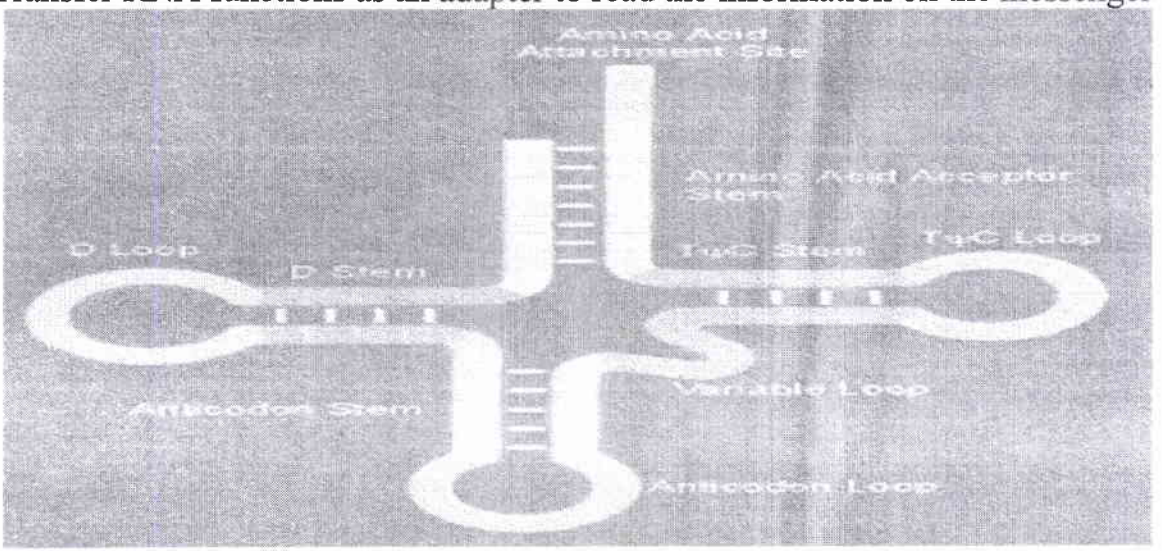
Gene Expression : translation

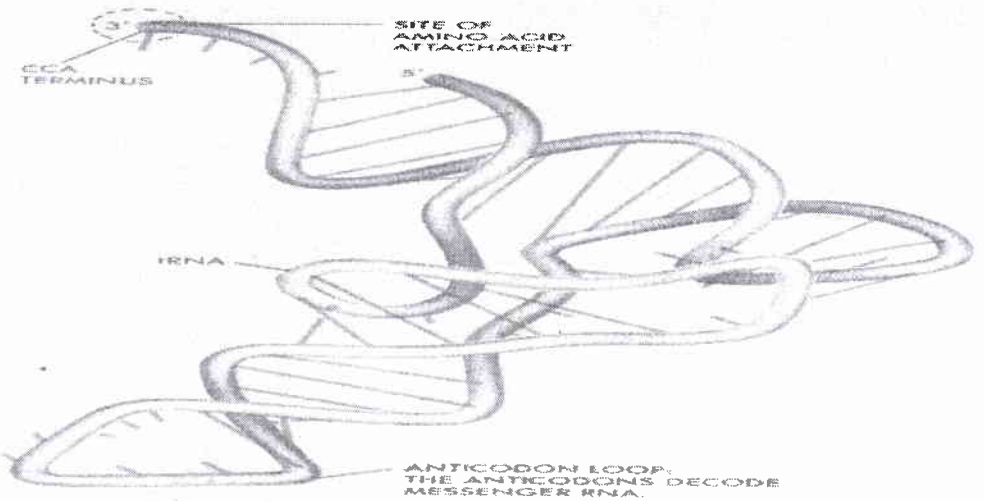
Once the information from a gene has been transcribed into an RNA molecule, the RNA serves as a messenger, transporting the information from the DNA to subcellular structures called ribosomes. Ribosmes are large ball-like structures, and it is here that the information in the messenger RNA is translated from the nucleotide language into the amino acid language. As a chain of amino acids is made, it spontaneously folds to form the protein specified by gene in the DNA.

The translation machinery works in the following way. Messenger RNA attaches to a ribosome near a site on the messenger called the start reading the message. In bacteria this begins before the messenger RNA and ribosomes can be attached to DNA; The messenger RNA leaves the DNA before attaching to ribosomes.

Meanwhile, the 20 different types of amino acids ,which eventually will be linked to form a protein, are floating free in the cell. Each amino acid is joined to another type of RNA molecule called transfer RNA (tRNA) .

Transfer RNA functions as an adapter to read the information on the messenger RNA .





The structure of a transfer RNA molecule. Base pairing within the single-strandd molecule gives it its distinctive shape. The anticodon loop is the portion that decodes messenger RNA. An amino acid attaches to the CCA bases at the 3' end of the chain.

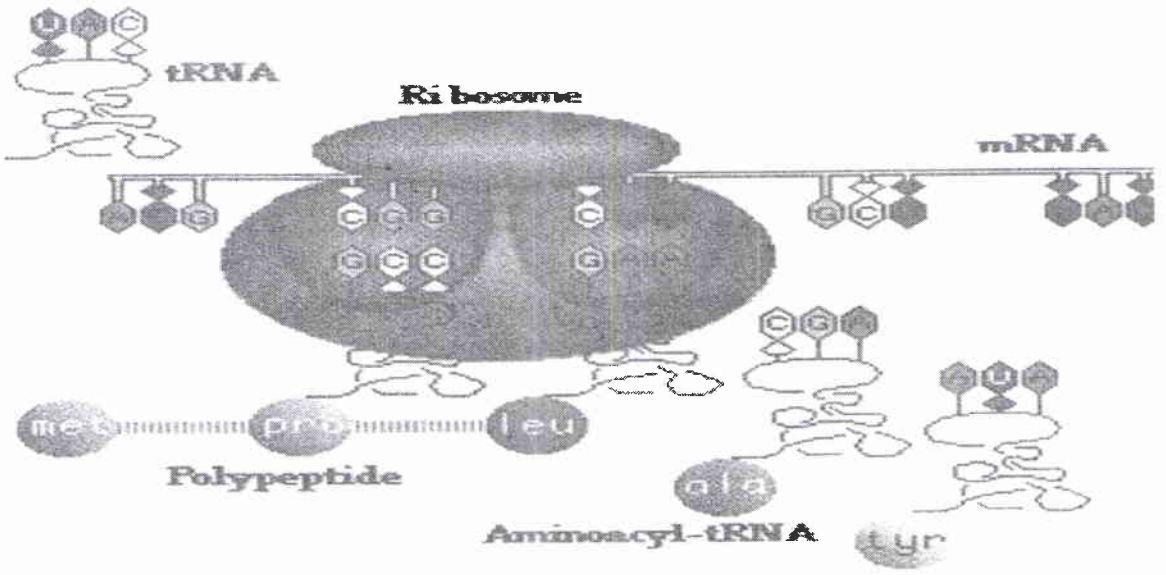
There are more than 20 different aminoacyl-tRNA synthetasea, at least one for each type of amino acid; each of these enzymes recognizes and attaches to only one of the 20 types of amino acid. Each enzyme is also able to recogenize and attach to a specific type of transfer RNA .

There is a different type of transfer RNA for each type of amino acid. Once a particular amino acid and a particular transfer RNA have attached to a particular aminoacyl-tRNA synthetase, the synthetase links the amino acid to the transfer RNA.The amino acid-trasfer RNA pair is then released from the enzyme. The net effect is to create 20 different amino acid-tranfer RNA pairs. Each of the transfer RNAs has a three-nucleotide region called an anticodon located opposite the end where the amino acid is attached.Each of the 20 types of transfer RNA has a different anticodon. Thus the particular amino acid at one end of the transfer RNA always corresponds to a specific set of three nucleotides in the anticodon region of the transfer RNA .

The specificity of the aminoacyl-tRNA synthetase ensures that this is the case. The anticodon on the transfer RNA can be exposed to form base pairs with the messenger RNA .

One particular transfer RNA has an anticodon triplet complementary to the start codon, or triplet,on messenger RNA.That transfer RNA and the messenger RNA lock together on the ribosome so that the two triplets; the codon on the messenger and the anticodon on the transfer RNA , form base pairs. This joining is governed by the complementary base-pairing rule: If the start codon on the messenger RNA is A-U-G , the only transfer RNA that will fit has an anticodon that reads C-A-U. The particular amino acid attached to this transfer RNA is destined to become the first link in the new protein chain.

GENE CLONING



In order to be replicated, DNA molecules must contain an origin of replication, and in bacteria and viruses there is usually only one per genome. Such molecules are called replicons. Fragments of DNA are not replicone and in the absence of replication will be diluted out of their host cells. It should be noted that even if the DNA fragment contains an origin of replication this may not functions in a foreign host cell. So that, the solution is to attach them to a suitable replicon. Such replicons are known as vectors or cloning vehicles. The basic steps for gene cloning are as follow:-

1. A fragment of DNA, containing the gene to be cloned and the vector DNA are cut with either same restriction enzyme or ones producing the same ends. The two samples are then mixed and ligated with DNA ligase enzyme to generat artificial recombinant DNA molecule.
2. The vector act as a vehicle that transports the gene into a host cell (transformation) , which usually is bacterium.
3. Within the host cell the vector multiplies , producing numerous identical copies not only of itself but also of the gene that it carries.
4. When the host cell divides, copies of the recombinant DNA are passed to the progeny and futher vector replication takes place.
5. After a large number of cell divisions, a coloy or clone, of identical host cell is produced. Each cell in the clone contains one or more copies of the recombinant DNA molecule, the gene carried by the recombinant molecule now is called cloned.

Cloning Vehicles

The central component of a gene cloning experiment is the vehicle, which transports the gene into the host cell and is responsible for its replication. To act as a cloning vehicle, a DNA molecule needs to display several features. Most important, it must be to replicate within the host cell, so that numerous copies of the recombinant DNA molecule can be produced and passed to the daughter cells. It also needs to be relatively small, ideally less than 10 Kilobases (kb) in size, as large molecules are more difficult to manipulate. Two kinds of DNA molecule satisfy these criteria : plasmids and bacteriophage chromosomes.

Plasmids:

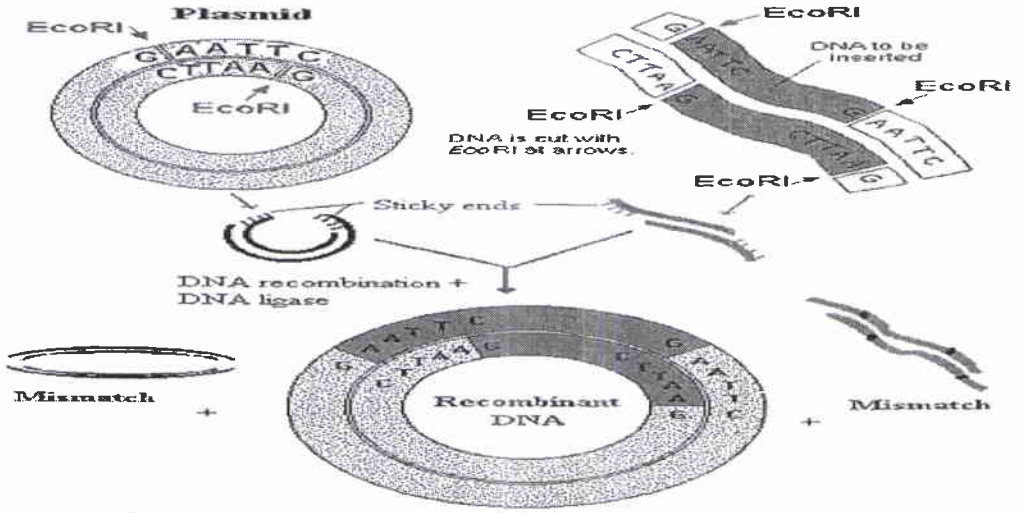
Plasmids are small circular extrachromosomal double stranded DNA , widely distributed throughout the bacterial cells. All plasmids possess at least one DNA sequence that can act as an origin of replication, so they are able to replicate independently of the cell chromosome.

They almost always carry one or more genes responsible for a useful characteristic displayed by the host bacterium. For example, The ability to survive in normally toxic concentrations of antibiotics such as chloramphenicol or ampicillin is often due to the presence in the bacterium of a plasmid carrying antibiotic resistance genes .

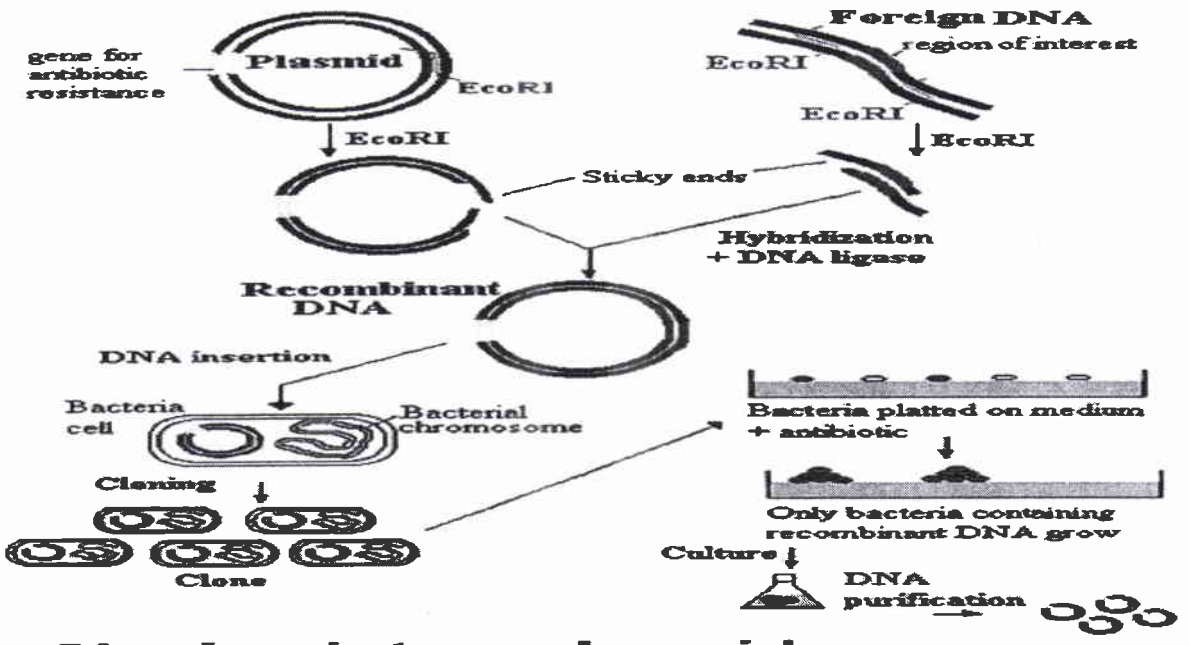
These resistance genes are usually used as a selecable marker to ensure that bacteria in the culture contain particular plasmid. Plasmids range from about 1.0 kb for the smallest to over 250 kb for the largest plasmids,so only few will be useful for cloning purposes.

The smallest plasmids make use of the host cell's own DNA replicative enzymes in order to make copies of themselves. A few types of plasmid are able to replicate by inserting themselves into the bacterial chromosome, called integrative plasmids or episomes. They are stably maintained in this form through numerous cell divisions, but will at some stages exit as independent elements .

Generally,a useful plasmids are those of low molecular weight , able to confer or more of selectable phenotypic traits on the host cells, present in the cell in multiple copies and having a single sites for a large number of restriction enzymes.



Inserting a DNA Sample into a Plasmid



Cloning into a plasmid

Bacteriophages (phages):

They are viruses that specially infect bacteria. Like other viruses , they consist merely of a DNA (or occasionally RNA)

Molecule carrying a number of genes, surrounded by a protected coat or capsid made up of protein molecules. The phages particles attaches to the outside of the bacterium and in injects its DNA chromosome into the cell.There are two infection cycles for phages; the lytic and lysogenic. The former is complete quickly, within 20 minutes and its associated with the ltsis of the bacterial cells . In contrast, the lysogenic cycle is characterized by retention of the phage DNA molecule ih the host bacterium. There are two main types of phage structure : (a) head- and- tail , (b) filamentous.Although there are many different varietes of baceriophages, only the lambda and the M13 phages have found real role as cloning vehicle.

(a) Lambda phages :

It is a head- and- tail phage of 49 kb in size. The linear molecule consists of two complementary strands of DNA, however, at either end of the molecule is a short 12 one complementary nucleotide stretch in which the DNA is a single strand. These two single strands can base –pair with another to form a circular, completely double stranded DNA molecule. These ends are referred to as 'sticky or cohesive ends" and called cos sites. The entire central region of the phage DNA is not essential for its replication in E. coli ,consequently it can be deleted or replaced without seriously impairing the infectious growth cycle.

The lambda phage can accomimodate about 5% more than its normal complement of DNA. Many vector derivatives of this phage have been created to accommodate large fragments of foreign DNA.This vector is widely used for the construction of genomic and cDNA library.

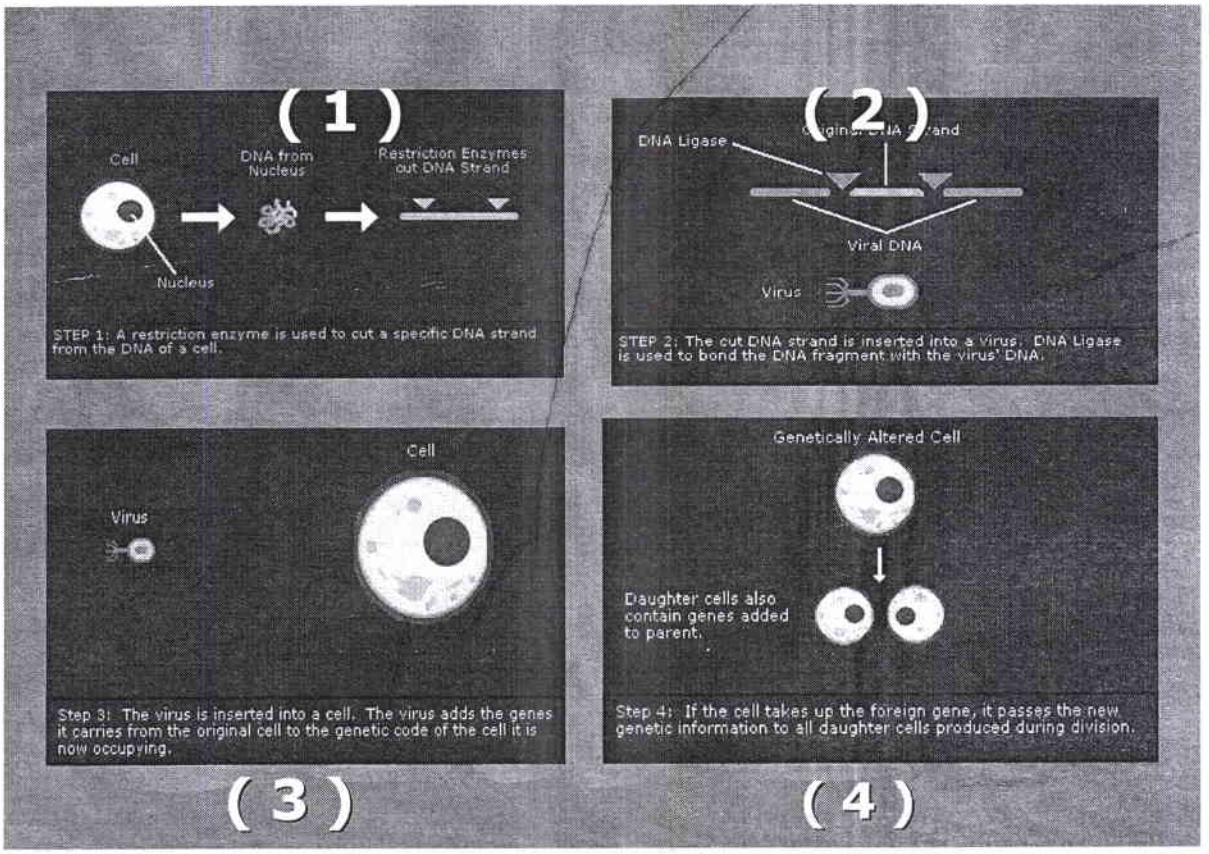
(b)M13 phages :

M13 is a typical example of filamentous phage and it is completely different in structure from the lambda phages. The M13 genome is about 6.4 kb, it is circular and consists entirely of single stranded DNA . Once inside the cell, the phage DNA acts as a template for synthesis of a complementary strand, resulting in normal double stranded DNA (replicative form). This form of M13 genome behaves very much like a plasmid and it is easily prepared from the infected bacterial cells and can be reintroduced by transfection. Most importantly, genes cloned with an M13 vector can be obtained in the form of single- stranded DNA which is needed for DNA sequences and in vitro mutagenesis.

Cosmids :They are plasmids which have been constructed including a fragment of lambda DNA "the cos site". They are suitable to clone large DNA fragments, so that cosmids are attractive vectors for constructing libraries of eukorytic genome fragments. Cosmide have selectable markers such as antibiotic resistance gene, and a plasmid origin of replication. They are able to produce colonies on selective media, just as with a plasmid vector.

Cutting and joining DNA Molecules.

Once pure samples of DNA have been prepared, the next step in gene cloning experiment is construction of the recombinant DNA molecule. To produce this recombinant molecule, the vector, as well as the DNA to be cloned, must be cut at specific points to open up the circle and then joined together in a controlled manner. A wide variety of enzymes have been developed since 1970 . So , DNA molecules can be shortened, lengthened, copied into RNA or into new DNA molecules, and modified by addition or removal of specific chemical groups. The DNA manipulative enzymes can be grouped into five broad classes depending upon the type of reaction that they catalyze.



1) Nucleases :

They are enzymes that cut, shorten or degrade DNA molecules.There are two distinct kinds of nucleases, exonucleases and endonucleases. The formal remove nucleotides at a time from the end of a DNA molecule. The later are able to break internal phosphodiester bonds group. The restriction endonucleases are the most important class within this group. The discovery of these enzymes was one of the key break throughs in the development of genetic engineering. These enzymes are synthesized by many perhaps all, species of bacteria; over 1200 different ones have been characterized.

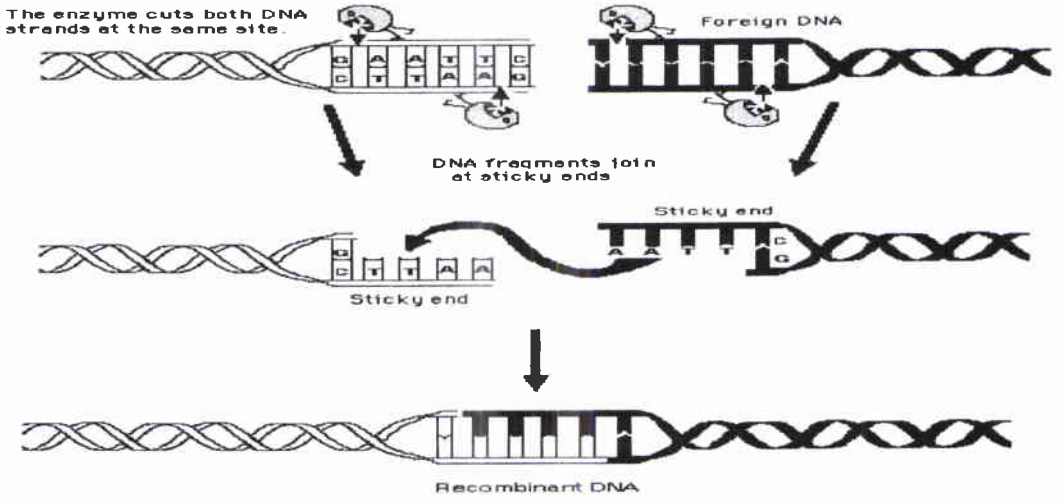
Three different classes of restriction endonucleases are recognized. Types I and III are rather complex and have limited role in gene cloning. However, type II restriction enzymes are so important in recombinant DNA technology. The vast majority of them recognize and break DNA within particular sequences of tetra, penta, hexa-, or hepta- nucleotides and nowhere else. For example, enzyme called *pvuI* (isolated from *proteus vulgaris*) cuts DNA at hexanucleotide CGATCG. In

contrast, a second enzyme from the same bacterium, called *pvuII*, cuts at a different hexanucleotide CAGCTG.

The exact nature of the cut produced by a restriction enzyme is of considerable importance in the design of a gene cloning experiment. Some enzymes cut the DNA in the middle of the recognition sequence, resulting in a blunt end.

However, a larger number of enzymes do not cut the two strands of DNA at exactly the same position. Instead the cleavage is staggered, usually by two or four nucleotides, so that the resulting DNA fragments have short single stranded overhangs at each end. These are called sticky or cohesive ends, as base pairing between them can stick the DNA molecule back together. Some enzymes may produce the same sticky ends, so their products can be joined back together.

Restriction Enzyme Action of EcoRI



2) Ligases :

DNA ligases are enzymes that join DNA molecules together. Within the cell the, enzyme is responsible for repairing single stranded breaks " discontinuities" that may arise in the double stranded DNA molecules during for example, DNA replication. All living cells produce DNA ligases, but the enzyme used in genetic engineering is usually from E.coli bacteria that have been infected with T4 phage.

3) Polymerases:

These are enzymes that synthesize a new strand of DNA complementary to an existing DNA or RNA template. Most of them can function only if the templatr

possesses a double stranded region which acts as a primer for initiation of polymerization. Examples of these enzymes are DNA polymerase I, Klenow fragment , reverse transcriptase and Taq DNA polymerase.

4) Modifying enzymes :

They are able to remove or add specific chemical groups to DNA molecules. For example, alkaline phosphatase, polynucleotide kinase and terminal deoxynucleotide transferase.

5)Topoismerases :

Enzymes that can introduce or remove supercoils from covalently closed circular DNA. They have yet to find a real use in genetic engineering.

LINKERS and ADAPTORS:

They are commonly used to put a stiky ends on to a blunt ended DNA molecules. Linkers are short pieces of double-stranded DNA,of knowen nucleotide sequence, that are synthesized in the test tube. It is blunt ended and contains a restriction site for enzyme that is able to generate a sticky end. Adaptors are like linkers, short synthetic oligonucleotides, but they have one blunt end and one sticky end. The idea is of course to ligate the blunt end of the adaptor to the blunt ends of the DNA fragment, to produce a new molecule with sticky ends.

Introduction of DNA into Living cells

Strictly speaking the word"cloning" refers only to this stage and not to the construction of the recombinant DNA molecule. Cloning can supply milligrams or even milionfold increase in yield of recombinant DNA. Most species of bacteria are able to take up recombinanat DNA molecules (plasmid and inserted gene)from the medium in which they grow.

E.coli is the most commonly bacteria used in gene cloning experiment. The bacteria have to undergo some physical and /or chemical treatment that will enhance their ability to take up DNA. Cells that that have undergone this treatment are said to be *Competent*. The processes of take up the DNA molecule is known as *Transformation.*

The bacterial cells are then usually transferred to nutrient medium to allow phenotypic properties conferred by the plasmid to be expreed. Finally, the cells are plated out on selective medium. The recombinant plasmids always carry selectable markers which allow us to detect the transformant cells.

Plasmids may have antibiotic resistant markers e.g. pBR322 which is resistance to ampicillin and tetracycline . Others like pUC8 plasmid carries the ampicillin

resistance gene and a gene called LacZ', which codes for enzyme B-galactosidase. This allow, to select the transformant cells according to their resistance to ampicillin and so the color development ; where the non-transformant clonies develp blue color and the transormant ones will be white.

Each transformant cell in the in the bacterial clonies contains copies of the recomninant DNA molecule,the gene carried by the recombinant molecule now is called cloned. The recombinant DNA molecule constructed with a phage vector can be introduced into bacterial cells by an equivalent process called transfection.

Organisms other than bacteria have also been employed for introduction of recombinant DNA like yeast, fungi, viruses, animals and plants. Viruses like vaccinia, baculovirus, bovine papillomavirus and retrovirus have been utilized to express foreign genes for vaccine and diagnosis purposes.

With animals and plants the desired end-product may not be transformed cells, but a transformed organism.Plants are relatively easy to regenerate from cultured cells. A single transformed plant,which will carry the cloned DNA in every cell and will pass it to its progeny. Animals cannot be regenerated from cultured cells, so obtaining transformed animals requires different approach.

The standard technique with mammals such as mice is to remove fertilized eggs from the oviduct, to microinjected DNA , and then to re-implant the transformed cells into the mother's reproductive tract.

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Practical part

Genomic DNA isolation procedures includes the following steps :

(1) Sample preparation

(2) Some form of cell lysis

(3) Deproteinization

(4) Recovery of DNA

(1) Sample Preparation

A) Whole organs

- Mincing , freezing in liquid nitrogen
- Crushing to produce digestible pieces
- Resuspension of powdered tissue in lysis-digestion or extraction buffer.

B) Tissue Culture Cells

- Spining suspension cultures out of their serum-containing media
- Trypsinization of adherent cells
- Recollection by centrifugation
- Washing cells in Tris buffer or PBS.
- Resuspension in the lysis-digestion or extraction buffer

C) Body Fluids (blood / plasma or serum , urine, CSF)

- Collection of whole blood in tubes containing anticoagulant ; EDTA.
- Centrifugation and remove the plasma and /or buffy coat separately in different tubes prior to DNA extraction

D) Other choices : plasma, serum preferred with extracellular target organisms.

- Centrifugation at 10,000 Xg for 10 minutes to remove the large particulates.
- Carefully remove this supernatant and pellet viruses by centrifugation at 50,000 rpm for 45 minutes in an SW 50.1 rotor.
- Remove and discared the supernatant, and dissolve the viral pellet in 500 ul of TE.

E) Body fluid samples

Can be used directly in the PCR process after boiling for 10 minutes at 100 °C.

Due to the viscosity of the sample

- Mixing with equal volume of digestion buffer, incubation for one hour and using the lysate as DNA template for PCR.

F) Pap Smears or Paraffin-Embedded Tissues

-Extraction of paraffin from the embedded tissues by xylene for one hour at room temperature.

G) From bacterial cells:

- Grow bacterial cells to desired density.
- Centrifuge 1.5 ml of cells in a microfuge for 5 minutes.
- Drain off the supernatant and resuspend the pellet in lysis buffer.

E) Clinical Swabs:

Cervical , vulvar, or penile samples are collected with a cotton swab (pre-wet with saline) or cytobrush. The specimen is placed in a 10-to 15- ml conical tube containing 2ml PBS plus a 2X concentration of fungi-Bact.If the sample is to be processed within 24 hours, it can be kept at room temperature. Refrigeration at 4 °C is recommended for longer storage times.

Pellet the cells by centrifugation for five minutes at 2000 to 3000 rpm and carefully remove the supernatant with an aspirator.

(2) Cell Lysis and Digestion

-Proteinase K

-Ethylene diamine tetraacetic acid (EDTA)

-Detergents

Ethylene diamine tetraacetic acid inhibits aggregation of DNA to each other and to proteins.

Detergents: promote dissociation of proteins from nucleic acids. **The ionic detergent** sodium dodecyl sulfate (SDS) promotes dissociation of lipid membrane structures and **the nonionic detergents** (tritonX100, NP-40, tween 20).

(3) Extraction of Genomic DNA

Most common method used for extraction: phenol/Chloroform-Isoamyl alcohol extraction in which Deproteinization of an aqueous phase containg the desired genomic DNA.

1. Resuspend the pelleted cells in cold cell lysis buffer

(10 mM Tris-HCL, pH 7.4 , 10mM sodium chloride and 10mM EDTA). Aminimum volum of 100 ul of lysis buffer should be used.

2. Add SDS to a final concentration of 1%. If SDS directly to the cell pellet prior to resuspension, large clumps of cells may form which are difficult to break up and may reduce the total yield of DNA.

3.Add Proteinase K to a final concentration of 200 ug/ml.

4. Incubate the reaction overnight at 56 °C with gentile rocking.

5. Extract with an equal volume phenol : chloroform :isoamyl alcohol (25 :24 : 1) . Centrifugation for 10 minutes The phases should be well separated, otherwise recentrifuge the sample.

6. Transfer the aqueous layer to a new tube. If the interphase is large, add an additional 1/4 volume of cell lysis buffer to the tube containing the phenol and the interphase and re-extract as described above.

7. Combine the aqueous layers and extract with an equal volume of chloroform: isoamyl alcohol (24:1).Centrifuge for 10 minutes in cooling centrifuge

8. Transfer the aqueous layer containing the DNA to a new tube.

(4) Recovery and purification of DNA

A) Precipitation of extracted genomic DNA (aqueous phase) by adding : 0.1 volume of sodium or ammonium acetate (0.2 -0.3 M final conc.) + 2.5 volumes of 100% cold ethanol.

B) Centrifugation to obtain DNA pellet.

C) Washing the pellet by 70% ethanol.

Resuspension of dried DNA pellet in TE buffer. (10mM Tris HCL, pH 7.6 and 1mM EDTA).Pancreatic RNAase frequently added to eliminate contaminating RNA.

DNA concentration and purity determined by spectrophotometry :

- Measuring the absorbance at 260 and 280 nm.
- Quantification : 1 OD at 260 = approx. 50 ug of dsDNA.
- The A at 260 /280 should be above 1.8 , a lower ratio is an indication of protein contamination.
- The extraction step should be repeated.

Extraction of DNA from Bacterial cells

The purification of DNA from bacteria can be accomplished by phenol extraction and ethanol precipitation , except that additional procedures should be included to break open the cell walls (i.e lysozyme digestion or using alkaline media)

1. Grow bacterial cells to desired density.
2. Centrifuge 1.5 ml of cells in a microcentrifuge for 5 minutes. Drain off the supernatant.
3. Resuspend the pellet in 200 ul STET buffer. (8% sucrose , 50 mM Tris-base, pH 8.0 , 50 mM EDTA , 0.1% Triton X-100).
4. Add 4ul of the 50 mg /ml lysozyme solution to the resuspended pellet and incubate at room temperature for 5 minutes, then at 94 °C for 1 minute.
5. Add SDS (10 %) to a final concentration of 0.5 % and Proteinase K to a final concentration of 100 ug/ml. Mix and incubate for 1 hour at 37 °C .
6. Add NaCl (4M) to the reaction, to a final concentration of 0.5 M.
7. Add 1/10 volum of the CTAB/NaCL (5% (w/v) cetyl-trimethylammonium bromide and 0.5 M NaCl)solution. Mix and incubate at 65 °C for 10 minutes.
8. Perform phenol extraction and ethanol precipitation .

Extraction of DNA from serum

Many viruses and bacteria are not cell associated , but occur free in the blood. The DNA from these organisms can be recovered from blood by preparing a serum or plasma fraction and extraction with phenol. The quantity of DNA contained in these sources is extremely low, so carrier DNA must be added prior to phenol extraction and ethanol precipitation to assure quantitative recovery.

1. centrifuge whole blood or clotted blood at 10.000 Xg for 10 minutes at room temperature. Remove the upper layer containing the plasma or serum.
2. add an equal volume of extraction buffer(150mM NaCl , 10 mM EDTA,10mM Tris-HCLpH 8.0,2% SDS and 5ug/ml salmon sperm DNA) to the serum or plasma and add proteinase K to250ug/ml.incubate at 37°C overnight.
3. Extract the sample with an equal volume of phenol : chloroform : Isoamyl alcohol (25:24:1). Incubate the emulsion at 50 °C for 20 minutes. Centrifuge for 10 minutes and transfer the aqueous layer to a new tube, leaving the interphase behind.
4. Add up to 1/2 of the original serum or plasma volume of extraction buffer to the tube containing the phenol and interphase and re- extract at room temperature. Centrifuge for 10 minutes .
5. Combine the aqueous layers from steps 3 and 4 add an equal volume of chloroform : soamyl alcohol and vortex. Centrifuge for 10 minutes and transfer the aqueous layer to a new tube.
6. Add 1/10 volume of 2 M sodium acetate plus 2 volumes of absolute ethanol to the aqueous layer. Incubate at -20°C overnight.
7. Centrifuge for 10 minutes. Wash the pellet with 70% ethanol, then centrifuge for 10 minutes.
8. Wash the pellet with 100% ethanol then centrifuge for 10 minutes.
9. Aspirate off the ethanol and air-drynthe pellet for 15 minutes. Resuspend the DNA in TE (10 mM Tris-HCl, pH 7.6 and 1 mM EDTA) to 1/2 the original volume of serum used.

Extraction of DNA from blood

Blood samples should be collected in EDTA (0.1 M final concentration) and mixed immediately.

1. Lable 1.5 ml microfuge tube with the blood sample number, add 500 ul TE buffer to each tube.
2. Add 100 ul blood to the labeled microfuge tube.
3. Vortex each sample to mix. Let stand at room temperature for 5 minutes. Centrifuge samples for 2 min. at maximum speed .

4. Discard the supernatant into a beaker containing 10% Bleach to avoid contamination.
5. Add another 500 ul of TE to the pellet. Vortex well until the pellet is mostly broken apart. Let stand at room temp. for 5 minutes. Centrifuge samples for 2 min. at maximum speed in microcentrifuge.
6. Repeat step 6 using 500 ul of RCLB buffer instead of TE. Pipette up and down to dissolve the pellet. Incubate 10 min. rocking at room temp. and centrifugation as in step (6).
7. After the last RCLB wash, remove and discard supernatant. Add 200 ul of prewarmed (56 °C) DSP buffer and vortex the samples.

   DSP buffer : 980 ul DSP stock solution

   15 ul proteinase K (10 mg/ml)

   5 ul Tween 20

   (proteinase K and Tween 20 must added fresh.)
8. Digest samples at 56°C for 6 hour. Vortex after one hour to mix.
9. Heat the samples at 90 °C for 10 min., to inactivate the PK.
10. Spin down the samples, use 2 ul of the supernatant as a DNA template for your PCR reaction.

Reagents and Buffers

TE buffer :

5.0 ml, 1 M Tris, pH 8.0

0.1 ml, 0.5 M EDTA, pH 8.0

Add water to 500 ml, autoclave and store at room temp.

RCLB buffer :

171.2 g sucrose

5.0 ml, 1 M Tris, pH 7.6

2.5 ml, 1 M MgCL2

Add water to 500 ml, autoclave and store at room temp.

50X TAE buffer:

242 g Tris-base

57.1 ml, glacial acetic acid

100 ml 0.5 M EDTA (pH 8.0)

Add water up to 1000 ml, autoclave and store at room temp.

Ethidium bromide:

10 mg / ml dd water

6X Gel loading buffer :

15% ficoll in water

0.25% xylene cyanol

0.25% bromophenol blue

Store at room temp.

Extraction of DNA and RNA from blood using guanidine thiocyanate and celite

(Diatomaceous Earth)

1.start with 2 mls of blood or approximately 800 ul of blood pellet.

2.Freeze pellet , then thaw at 37 °C .

3.Add 1 ml of TE pH 8.0, invert to mix then centrifuge at 14.000 rpm for 5 minutes.

4.Discared supernatant by suction. Repeat steps 2 , 3.

5.Add 900 ul of L6 buffer and 60 ul of Celite , vortex for 10 seconds.

6.Incubate at room temperature for 10 minutes.centrifuge at 14.000 rpm for 15 seconds.

7. Discard supernatant by suction.

8. Add 1 ml of L2 buffer, vortex for 10 seconds, centrifuge at 14.000 for seconds and discard supernatant by suction. Repeat this step twice.

9. Add 1ml of 70% ethanol, vortex for 10 seconds centrifugation at 14.000 rpm for 15 seconds then discard the supernatant by suction. Repeat twice.

10.Add 1 ml of acetone , vortex for 10 seconds. Centrifugation at 14.000 rpm for 15 seconds then discared the supernatant by suction.

11. Place open tube into speedvac. Spin with vacuum on and heat set to medium for 15 minutes.

12.Add 110 of TE pH 8.0 and vortex then elute in covered tubes in a water bath at 56 °C for 10 minutes. Then vortex for 10 seconds.

13. Centrifugation at 14.000 for 2 minutes.

14. Transfer supernatant to siliconized 1.5 ml centrifuge tube and freeze at

-70 °C.

Reagents:

Diatomaceous earth :

20 g Diatomaceous earth

100 mls DEPC water

1 ml HCL (32 % w/v)

Divide into 5 ml aliquots . Close tightly and autoclave at 121 °C for 20 minutes. After autoclaving, open caps and allow to cool. Retighten caps. Sable in the dark for 6 months at room temperature.

L6 Lysis Buffer:

120 g Fluka guanidine thiocyanate

100 mls 0.1 M Tris- HCL pH 6.4

22 mls 0.2 M EDTA pH 8.0

Heat to 65 °C in water bath then add 2.6 g Triton X-100 , allow to cool then ph to 8.0 with 10 N NaOH, place in dark bottle. Stable at room temp. For 3 weeks only.

L2 Washing Buffer:

120 g Fluka guanidine thiocyanate

100 mls 0.1 M Tris- HCL pH 6.4

Heat to 65 °C. Place in dark bottle. Stable at room temp. For 3 weeks only.

Mini- prep in ten minutes

This is a modified alkaline lysis method that does not require phenol-chloroform extractions or lysozymes. It has been found to be satisfactory for further use in enzyme digestions without additional reagents.

1. pellet 1.5 ml of overnight culture for 10 sec. in a microfuge.
2. Gently decant the supernate leaving 50-100 ul together with the pellet. Vortex the sediment at high speed to resuspend the pellet completely. In order to resisted the cells completely 50-100 ul should be left. If more is left, dilution of the TENS buffer in step 3 may result.
3. Add 300 hl TENS , vortex 2 sec. until the mixture becomes sticky. If more than 10 min are needed before moving to the next it is best to keep the samples on ice to prevent the degradation of chromosomal DNA which may coprecipitate with plasmid DNA in steps 6 and 7.
4. Add 150 ul of 3M sodium acetate pH 5.2 and vortex 2 sec. to mix completely.
5. Spin 2 min. to pellet the cell debris and chromosomal DNA.
6. Transfer the supernate to a fresh tube. Add 900 ul 100% ethanol which has been cooled.
7. Spin for 2 min. to pellet the plasmid DNA and RNA . A white pellet will be seen.
8. Discard the supernate, rinse the pellet twice with 1 ml of 70% ethanol and vacuum dry the pellet.
9. Resuspend the pellet in 40 ul of TE buffer.

Reagents :

TENS buffer 500 ml (TE buffer containing 0.1 N NaOH and 0.5% sodium dodecyl sulfate)

Tris 5ml of 1 M stock solution	final Conc. 10mM
EDTA 1ml of 0.5M stock solution	
Then add	pH to 7.5
NaOH 2 gm	0.1 N
SDS 2.5 gm.	0.5%

Bring total volume to 500 ml.

This procedure was derived from " MiniPrep in Ten Minutes", Chen Zhou, Yujun Yang and Ambrose Y. Jong. Bio Techniques Vol. 8, No. 2 (1990) pg 172-173.

Preparation of Agarose Gel. (100 mls)

Note: Volume will vary for different gel sizes. Percentage agarose will vary , depending on size of DNA to separate.

Prepare: 10X TBE (electrophoresis buffer)

108 .0 gm Tris

55.6 gm Boric acid

4.2 gm Disodium EDTA

Make to 1 liter

Ethidum bromide (10mg/ml):

Note : ware gloves when handling ethidium bromide. Carcinogenic.

5X sample buffer:

0.025 gm bromophenol blue

o.o25 gm xylene cyanole

5.0 ml glycerol

5.0 ml sterial 0.1 M EDTA pH 8.0

Preparation of gels (0.8%) :

1. weight out o.8 gm of electrophoresis grade agarose. Dissolve in 100 ml of 1X TBE on hot plate.will need to boil for several minutes to ensure no undissolved particles remain.
2. When agarose is dissolved, stand fiask in 65 °C water bath for 30 mins.

3. After 30 mins. In the water bath , add 5ul of 10 mg/ml ethidium bromide stock soln., mix.
4. Pour solution into taped , flat, clean, electrophoresis tray, position comb immediately while still molten. Pay particular attention to elimination all bubbles, especially around the combs. Combs must be at –ve end.
5. When gel has (45 min.) remove tape and put in electrophoresis apparatus. Fill apparatus to 3mm above surface of gel with 1X TBE.
6. Remove sample comb. Add samples for electophoresis to wells. Samples should be 1X in sample buffer.
7. Apply current. Run 100 V constant voltage. Can run overnight by decreasing voltage to ~ 20 V. Will run towards +ve electrode.
8. Take photograph of gel with Polaroid camera under UV light.

Polymerase Chain Reaction

PCR master mix for ten samples 50 ul total volume reaction :

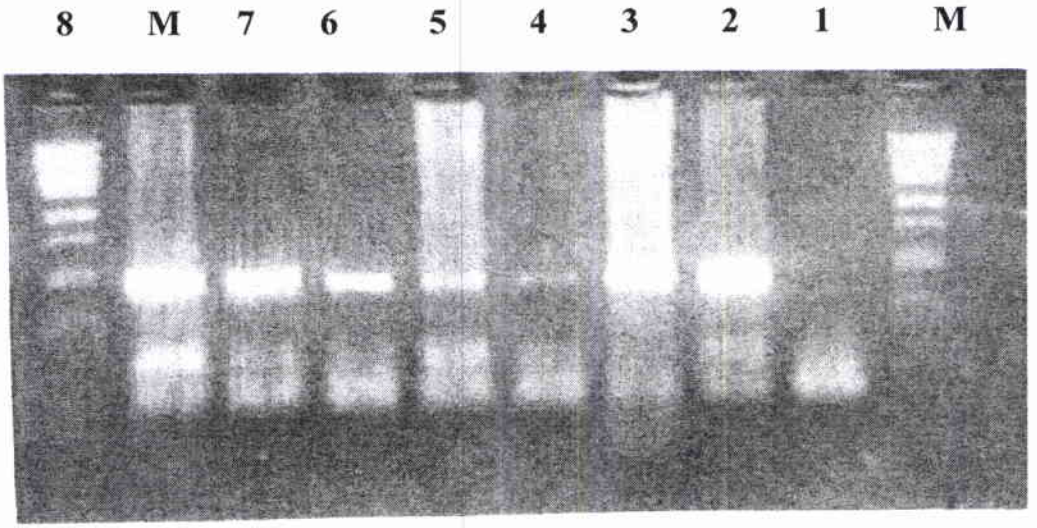
10 X 13.8 ul	dd water	138 ul
10 X 2.5 ul	10X PCR buffer	25 ul
10 X 5.0 ul	dNTPs (200 uM)	100 ul
10 X 0.5 ul	Primer #1 (10 pmol/ul)	5 ul
10 X 0.5 ul	Primer #2 (10 pmol/ul)	5 ul
10 X 1.5 ul	MgCl2 (1.5 mM)	15 ul
10 X 0.2 ul	Taq polymerase (1 unit)	2 ul

Take 48 ul PCR master mix . Add 2 ul DNA template to the PCR mix. Add 1 drop of mineral oil to each reaction.

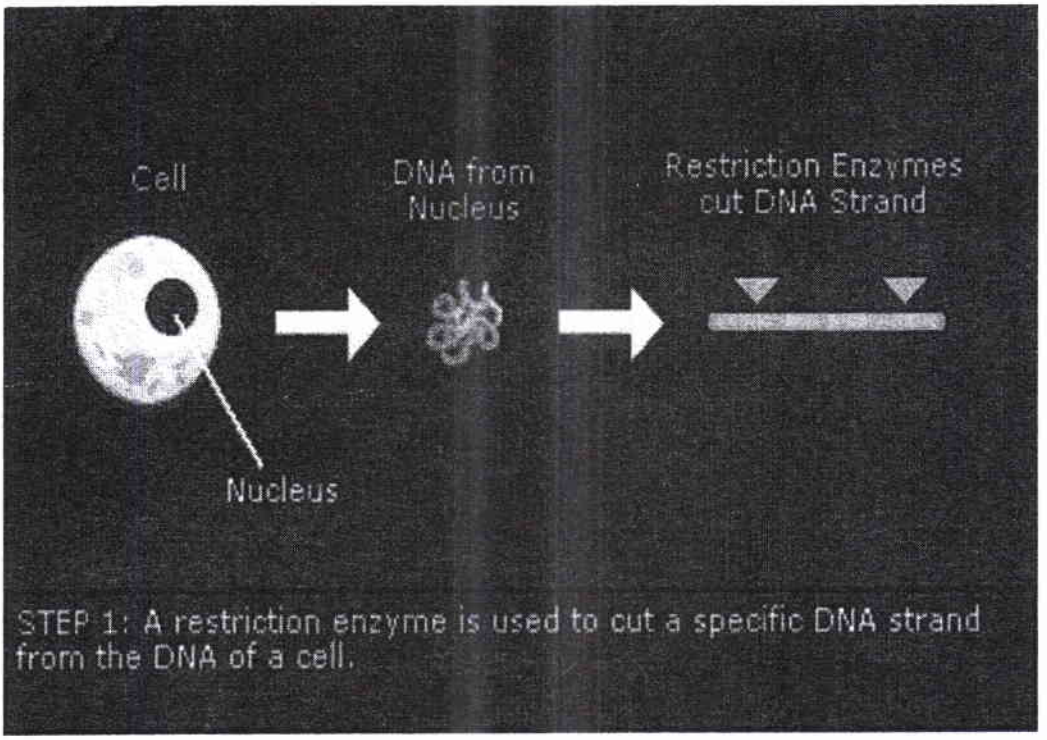
PCR program :

Denaturation	**93 °C**	**10 min.**	**prog # 001**
35 Cycles of :			**prog # 002**
Denaturation	**93 °C**	**1 min.**	**step.01**
Annealing	**49 °C**	**1.50 min.**	**step.02**

Extension	**72 °C**	**3 min.**	**step.03**
Final Extension at 003	**72 °C**	**10 min.**	**prog #**
Soak at 004	**4 °C**	**indefinite**	**prog #**



Ethidium bromide stained agarose gel electrophoresis of RT-PCR extracted from cattle infected with foot and mouth disease virus isolated from different localities.The amplified bands of 500 base pair (arrow) belong to ID gene(coding for part of Virus Protein I ,VPI surface protein (M). referred to 100 base pair marker. Lanes(1,4,6&7) showed amplified bands from infected cattle.Lanes(2,3&5) control positive samples.Lane(8) Control negative.The lower bands in all samples are primer dimer.



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Bacteriological Diagnosis of Brucellosis in From Animals

Bacteriological Diagnosis of Brucellosis in Farm Animals

By

Dr. Ashraf Ezz El-Deen Sayour

Researcher (Veterinary Bacteriologist & Immunologist)

Significance of *Brucella* identification & biotyping

* **Diagnostic importance:**

- **The only definitive means for proving *Brucella* infection:**

Bacteriological isolation, identification and typing of the causative agent helps a great deal in proving the infection status in problem herds as well as in areas where brucellosis is diagnosed for the first time. Serological diagnosis allows up to 90-95% accuracy where it may give some false negative and false positive cases

- **Monitoring and evaluation of serological tests:**

It is necessary to support the serological results with bacteriological examination whenever possible. False positive serological results may emerge every now and then due to natural agglutinins, crossreactions with similar Gram-negative bacteria and postvaccinal titres following imuunization with smooth strains. The sensitivity of immunoassays is measured by comparison with positive bacteriological results.

- **Differentiation between infected and vaccinated animals:**

Bacteriological examination decides 100% infection or 100% vaccination only when either a field strain or a vaccinal strain is recovered and identified.

* **Epizootological importance:**

Outbreak investigations are crucial to confirm and delineate the patterns of transmission of brucella strains, to test hypotheses about the sources and vehicles of transmission of these strains and to monitor the reservoirs of organisms. The involvement of a particular *Brucella* isolate in a disease outbreak can be documented, and the tracing of isolates within a country or between countries is made possible. For instance, it is essential sometimes to determine that purchased cattle or reentry of bred heifers into the herds was probably responsible for introducing infection and that the biovar was not readily transmitted to adjacent herds/ areas. Moreover, one of the major determinants for selecting the vaccinal strain is the predominant *Brucella*

species. Biotyping also contributes to epizootological surveillance and evaluation of control measures by documenting the prevalence over time.

* **Epidemiological importance:**

Detection of the *Brucella* species/ biovar(s) causing human infections is necessary. *Br. melitensis* is the most virulent *Brucella* species to both humans and animals followed by *Br. suis* and *Br. abortus* respectively. *Br. canis* can cause occasional human infection. It is crucial sometimes in the treatment of Malta fever patients to specify the infecting *Brucella* strain for selecting the antibiotic course of choice. For instance, streptomycin should be excluded from the treatment of infection with the vaccinal strain *Br. melitensis* Rev.1 where this strain is resistant to it. Likewise, rifampicin should be avoided in case of *Br. abortus* strain RB51 infection.

Taxonomic Background

Taxonomic Background

By Dr. Ashraf Ezz El-Deen Sayour

Taxonomic niche of the genus *Brucella*

For many years the genus *Brucella* was included with other phenotypically similar genera as *Bordetella*, *Pasteurella* and *Francisella* in the family *Brucellaceae*. DNA homology studies have proven that genus *Brucella* is unrelated to the other family members. In fact, most of the genera in the family were unrelated to each other. Subsequently, the *Brucella* group of microorganisms remained as a free floating genus of uncertain affiliation.

Finally, it is now accepted that *Brucella* belongs to rRNA superfamily IV as determined by DNA-rRNA hybridization. This superfamily includes genus *Brucella* and plant pathogens in the *Agrobacterium-Rhizobium* complex featuring the genera *Agrobacterium*, *Rhizobium*, *Mycoplana* and *Phyllobacterium* as well as the opportunistic human bacteria *Ochrobactrum anthropi* (formerly Centers for Disease Control, CDC, group Vd.) found in blood, respiratory tract, urogenital tract and urine.

Taxonomic subdivisions within the genus *Brucella*

The genus *Brucella* includes closely related bacteria. The genetic homology among members of *Brucella* is reflected by 57.9-59.2% G+C content of DNA. Another more discriminating test of genetic similarity is DNA-DNA hybridization. It revealed that the degree of relatedness for the DNA of all *Brucella* spp. is 96±4% barely permitting the differentiation to the species level only. On this basis, members of *Brucella* were considered as variants of a single species, *Br. melitensis*, with the currently recognized nomen-species identified as biovars. Nevertheless, there are some phenotypic differences do exist in the biochemical reactions, cultural characteristics, phage susceptibility and pathogenic properties of the *Brucella* members, as well as molecular evidence of some genomic variation. These mostly phenotypic variations constitute the recognized subdivisions within the genus and are correlated with animal host preference.

Generally speaking, in the taxonomy of any other genus, species behaving like those of *Brucella* would be treated as biovars or phagovars. Although there is taxonomic evidence for adopting the new system of classification, the current system reflects biological diversity crucial to keep with the polyphasic approach of defining bacterial species where phonetic, genetic, phylogenetic and ecological data should be involved. The host range of *Brucella* species classified them into

genetic groups evolutively linked to their favorite hosts. The presence of species-specific gene markers made the species biologically vital groups not just pathovars.

Routine identification to the genus level

Before proceeding with the methods for identification at the species and biovar levels, it must first be established that an isolate belongs to the genus *Brucella*. A trained *Brucella* bacteriologist can recognize *Brucella* cultures on the basis of colonial and cell morphology as well as slide agglutination before pure culture can be biotyped. To make the long story short, presumptive routine identification is done in the following order.

1. **Colonial morphology:** Smooth colonies are circular, convex, 1-3 mm in diameter with smooth glistening surface. They give a transparent honey colour in transmitted light and a bluish-white translucent appearance in reflected light.

2. **Microscopic appearance:** Heat-fixed smears of suspected *Brucella* colonies should be tested microscopically by the Gram's staining technique and the modified Ziehl-Neelsen's method.

3. **Test for dissociation:** This identifies the colonial phase as smooth, intermediate or rough. The colonies of organisms having *Brucella* morphology should be tested for dissociation by suspending the colonies in 0.1% aqueous acriflavin.

4. **Agglutination with *Brucella* antisera:** Suspected colonies should be screened for agglutination with *Brucella*-positive antisera.

5. **CO_2 requirement:** The supplementary CO_2 required by strains of *Br. abortus* and *Br. ovis* for growth is used as a nutritional factor and not simply to lower oxygen tension or pH. It is incorporated directly into their structural pyrimidines, glycine and alanine. Although the CO_2 requirement is a differential characteristic among *Br. abortus* biovars, it is not always stable. Duplicate agar slopes are incubated at 37° C; one in air and the other in an atmosphere with 10% CO_2. Slopes are compared for growth. If only few colonies grow in air, these should be ignored.

6. **Test for motility:** Suspected colonies are subcultured into 2 tubes of brucella broth. One of these is incubated at 37° C in air with 10% CO_2 and the other at 22° C in a similar atmosphere. Both cultures are then examined for motile organisms by the hanging drop technique. Motile organisms are not brucellae.

7. **Culture onto blood and MacConkey agar:** Cultures are streaked onto sheep blood agar and MacConkey blood agar plates and incubated at 37° C in air plus 10% CO_2. On blood agar, *Brucella* produce non-hemolytic greyish-white glistening colonies 0.5-1 mm in diameter. On MacConkey agar, little or no growth may occur after 5 days incubation. Rapidly growing, lactose-fermenting colonies are not brucellae.

8. **O_2 requirement:** *Brucella* strains will not grow under strictly anaerobic conditions, nor will they produce easily visible colonies in 24-48 hours when incubated at room temperature. Replicate culture plates should be incubated at 37° C in an anaerobic jar and aerobically at room temperature (18-22° C).

9. **Catalase test:** Catalase is a bacterial enzyme that splits the lethal H_2O_2, formed as an oxidative end product of aerobic carbohydrate metabolism, into H_2O and O_2. The test differentiates catalase-positive from catalase-negative bacteria. Heavy suspensions from cultures grown on blood-free medium are tested with catalase reagent (3% H_2O_2 10 volumes). Bubble formation or foaming is a positive reaction. A negative reaction will exclude brucellae.

10. **Oxidase test:** Cytochrome oxidase produced by some bacteria can reduce a redox dye (tetramethyl-*p*-phenylenediamine) used in the test giving deep purple colour. A loopful of culture grown on blood-free medium is smeared across a piece of filter paper impregnated with 1% of the dye solution. Most *Brucella* strains will give a positive reaction but *Br. ovis* and *Br. neotomae* give negative reactions.

11. **Urease test:** Urease produced can split urea (in Christensen's urea agar) to ammonia (alkaline) which gives magenta (purple pink) colour in the presence of phenol red indicator. A Christensen's agar slope is inoculated with a loopful of culture suspension, incubated at 37° C and examined after 15 minutes and 1, 2 and 18 hours.

12. **Nitrate reduction test:** Nearly all brucellae except *Br. ovis* and some strains of *Br. canis* are nitrate-positive.

13. **Glucose fermentation test:** The production of acid or gas in glucose-peptone water excludes *Brucella* strains.

Identification to the species level

I. Phage lysis tests:

There is a number of bacteriophages active only upon members of genus *Brucella*. Being so specific, these phages are of great taxonomic value for identification at both genus and species levels. On the basis of their *Brucella* host range, the phages are classified into 7 groups **(Table 1)**.

Oxidative metabolic tests:

The metabolism of *Brucella* is oxidative rather than fermentative. For each *Brucella* species there is a typical pattern of O_2 consumption on certain substrates. The ability of *Brucella* spp. to oxidize these substrates is measured with the Warburg respirometer. The results are reported as $QO_2(N)$ representing the microlitres of O_2 uptake per melligram of N_2 per hour minus the endogenous respiration rate of the organism. A value of $QO_2(N) > 50$ is positive indicating substrate utilization.

Table 2: The oxidative metabolic pattern of *Brucella* species

Brucella species	*Biovar*	*Group I*			Group II						Group III			
		Amino acids			Carbohydrates						Urea cycle amino acids			
		L-alanine	L-asparagine	L-glutamic acid	L-arabinose	D-galactose	D-ribose	D-glucose	i-erythritol	D-xylose	L-arginine	L-ornithine	L-citruline	L-lysine
Br. abortus	all	+	+	+	+	+	+	+	+	-	-	-	-	-
Br. melitensis	All	+	+	+	-	-	-	+	+	-	-	-	-	-
Br. suis	1,2	-	-	+	+	+	+	+	+	+	+	+	+	+
	3,4	-	-	+	-	-	+	+	+	+	+	+	+	+
	5	-	+	+	-	-	+	+	+	+	+	+	+	+
Br. neotomae		-	-	+	+	+	+	+	+	-	-	-	-	-
Br. ovis		+	+	+	-	-	-	-	-	-	-	-	-	-
Br. canis		-	-	+	-	-	+	+	+	-	+	+	+	-

Identification to the biovar level

- **CO_2 requirement**
- It was previously discussed in the genus identification.
- **H_2S production**
- Strips of lead acetate paper are placed on the mouth of the agar slopes without touching the agar surface. Lead acetate papers are examined daily for few days. The presence or absence of blackening is recorded and the strips are renewed daily. Blackening produced on only one day is considered negative.
- **Sensitivity to dyes**
- This detects the ability of *Brucella* biovars to grow in the presence of thionin and basic fuchsin. Dye plates are quartered to permit 4 samples to be tested on one plate. A loopful of the culture suspension is used to inoculate each quarter with 5 consecutive streaks without recharging the loop. Plates are incubated in air plus 10% CO_2 for 2-4 days. If growth appears on 3 or more streaks, the organism is regarded as resistant to this dye. Growth on two streaks or less is taken as a negative result.
- **Agglutination with polyclonal monospecific antisera**
- Drops of A, M and R antisera are mixed each with similar drops of culture suspension on a slide, rocked gently for one minute and examined for agglutination. Sometimes it is necessary to use the quantitative tube agglutination when culture is positive to both A and M antisera.

Table 3: Biovar differentiation of the species of the genus *Brucella*

Brucella species	*Biovar*	CO_2 req't	H_2S prod'n	Dyes: Thionin	Dyes: Fuchsin	Antisera: A	Antisera: M	Antisera: R
Br. abortus	1	+	+	-	+	+	-	-
	2	+	+	-	-	+	-	-
	3	+	+	+	+	+	+	-
	4	+	+	-	+	-	+	-
	5	-	-	+	+	-	+	-
	6	-	-	+	+	+	-	-
	9	+/-	+	+	+	-	+	-
Br. melitensis	1	-	-	+	+	-	+	-
	2	-	-	+	+	+	-	-
	3	-	-	+	+	+	+	-
Br. suis	1	-	+	+	-	+	-	-
	2	-	-	+	-	+	-	-
	3	-	-	+	+	+	-	-
	4	-	-	+	-	+	+	-
	5	-	-	+	-	-	+	-
Br. neotomae		-	+	-	-	+	-	-
Br. ovis		+	-	+	-	-	-	+
Br. canis		-	-	+	-	-	-	+

Buffered Acdified Plate Antigen Test

(BAPAT)

Buffered Acidified Plate Antigen Test (BAPAT)

BY

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Hess (1953a,b) reported that agglutination can be immunologically non-specific due to non- specific agglutinins which can agglutinate a variety of unrelated bacterial antigens. These cause about 60% of non -specific agglutination reaction to brucellosis. Agglutination may be immunologically specific but diagnostically non-specific. The usual reason for this is prior vaccination specially with the attenuated *Br. abortus* strain 19. before 8 months of age.

Rose (1955) ,and Rose and Roepke (1957) were the first who used a low pH buffered system to carry out preliminary trials for differentiating specific and non specific reactions in bovine Brucellosis using an acidified antigen.

Lambert and Amerault (1962) mentioned that a relatively high percentage of the low level seroagglutination reactions (doubtful or suspected) that were caused specifically by *Br. abortus* were inhibited by the increased acidity of the "Buffered Acidified Plate Antigen test" (BAPAT) and concluded that acidified plate antigens have little value as a supplemental test to determine the brucellosis status of suspected animals in infected or problem herds.

Nicolletti and Muraschi (1966) concluded that The buffered acidified plate antigen test (BAPAT) detects infection prior to CFT .

Nicoletti (1969) reported that data indicated that buffered acidified plate antigen test (BAPAT) detected 95% of the cultured positive cattle in infected herds,

Christine and Boyes (1971) concluded that in sera of a small group of cattle that had been experimentally infected four years earlier with a virulent strain of *Brucella abortqs*, at least three classes of antibodies were found; IgM, IgG1 and IgG2. Only IgG1 or IgG2 and IgM agglutini s were detected in sera of cogs that had ceased to shed viable *Br. abortus* organisms in their milk, whereas sera of chronic shedders showed a high content of IgG1, and lesser amounts of IgG2 and IgM antibodies. Typical agglutination patterns were produced by IgM and IgG2 antibodies but "a prozone" behavior was exhibited by IgG1 immunoglobulins. These prozoning properties of IgG1 were not evident until after the serum had been heated at 60 °C as reported by **Allan et al., (1976).**

Beh (1973) reported that purified preparations of IgG1, IgG2, IgA, IgM and low molecular weight antibodies were obtained from serum and tested for Brucella antibody activity in a variety of commonly used diagnostic tests. Antibodies in all preparations except IgM exhibited antiglobulin enhancement. In contrast to IgG1, neither IgG2 nor IgA fixed complement, (this mean that IgG1 fix complement but IgG2 and IgA do not fix complement) and IgM fixed complement at titres much lower than those obtained in the direct agglutination test. Antibodies in IgM and IgA preparations loose most of its agglutinating activity after heating at 65 °C for15 min.

Elberg (1973) reported that infection even in the chronic phase stimulates the production of IgG1 while residual vaccination titres are attributed mainly to IgM which is responsible for all non specific agglutination.

Angus and Barton (1984) developed and evaluated a buffered acidified plate antigen of pH 4.0 to be used in BAPAT as a presumptive test to reduce the non-specific reaction to the agglutination test. They also reported that BAPAT was slightly more sensitive than RPBT. The test was first introduced to Egypt via the American Project in 1986 and **Hamdy (1989)** did the first work on cattle.

Stemshorn et al. (1985) examined 105 bovine sera from Brucella–free herds esing different serological tests. The specificity of BAPAT was (98.8%) and the sensitivities of BAPAT was (75.4%), The authors recommended the use of BAPAT as a presumptive test for its high specificity and sensitivity together with a specific confirmatory test such as CFT

Dohoo et al. (1986) revealed that there was a good agreement between BAPAT and SAT .The authors also recommended that BAPAT could be used as a screening test .

(Alton et al., 1988), (Lambert, and Amerault, 1962 and Wright and Nielsen., 1990).stated that the BAPAT test detected a higher number of positive as compared with other serological tests and this attributed to the final pH of a serum-antigen mixture of 4.02±0.04 this pH enables some of the IgM, beside, IgG1, IgG2 and IgA to share in the reactions The high sensitivity of BAPAT if compared to that of RBPT may attributed to the amount of serum used in BAPAT (0.08 ml) is greater than the amounts of serum used in the RBPT (0.03 ml). On other hand, the relatively acidic pH of the RBPT (3.65) permits lesser amounts of IgM to share in the reaction, since this class of immunoglobulin is known to be acid labile **(Allan et al., 1976).**Being slightly more acidic than the BAPAT, the RBPT inhibited more IgM.

Hamdy (1989) in Egypt, examined a total of 1054 blood samples taken from different dairy cow farms. The incidence of infection was 40.8%, 38.7%, 38.8% and 38.43% by BAPAT, RBPT, Riv.T and TAT respectively and concluded that BAPAT gave highest positive reactors. 63 Brucella isolates were recovered from infected cows (57 from milk and 6 from aborted foeti). All the isolated strains were Br. melitensis biovar 3.

The author recommended the application of Br. melitensis Rev 1 vaccine in sheep and goat as a corner stone for eradication of the disease in Egypt .

Refai (1989) in Egypt mentioned that Br. melitensis biovar 3 and Br. abortus biovar 7 were the most frequent among the isolates and the author decided to use the buffered acidified plate antigen test (BAPAT) as a presumptive test. Positive samples are then tested with the tube agglutination and Rose Bengal (card) tests. For confirmation, Rivanol and CFT were used. A high incidence of Br. melitensis infection among sheep and goats is alarming as a source of infection to cattle.

Russell (1989) reported that false negative results are possible with all tests with sensitivity <100%. Moreover exposed animals in the incubative stage not having seroconverted at the time of testing are the most likely explanation that a heifer infected early in life develops a latent infectious disease called "heifer syndrome" and seldom seroconverted until first calving.

MacMillan (1990) mentioned that any visible agglutination in the BAPAT was considered as positive .

Wright and Nielsen, (1990). reported that the RBPT detected the lowest number of positive as compared with BAPAT (buffered acidified antigen plate test)and may be attributed to the final pH of a serum-antigen mixture in BAPAT is 4.02 + 0.04 . This pH enables some of the IgM, besides; IgG1, IgG2, and IgA to share in the reactions. In addition, the amounts of serum (antibodies) used in the BAPAT (0.08 ml) is greater than the amounts of serum used in the RBPT (0.03 ml.) On the other hand, the relatively acidic pH of the RBPT (3.65) permits lesser amounts of IgM to share in the reaction, since; this class of immunoglobline is known to be acid labile (Allan et al., 1976).Being pH of the RBPT slightly more acidic than the BAPAT, the RBPT inhibited more IgM.

OIE., (1992) reported that buffered brucella antigen tests are suitable for screening herds and individual animals

(Mia et al., 1992 and, El-Gibally et al., 1995) concluded that, TAT, RBPT and BAPAT were highly sensitive presumptive tests while CFT is specific confirmatory test. This conclusion was highly augmented by previous work of many workers.

Essam (1993) recorded that RBPT is still lower than BAPAT due to pH 3.65 of an antigen of Rose Bengal destroy IgM present in serum.

Abdel Hafeez et al. (1994) reported that the positive reactors percentage by BAPAT and RBPT were 38% and 23% among cattle. These findings may be due to the fact that RBPT antigen has acidity of pH 3.65.This lower pH of RBPT antigen inhibits the activity of IgM and detects IgG (enhance the agglutination). BAPAT has pH 4 which permits the detection of IgM and IgG. This explains why BAPAT is more sensitive than RBPT.

Farid et al. (1995) examined sera from 119 Friesian cows infected with Br. melitensis biovar 3 as well as 139 Friesian cows infected with Br. abortus biovar1. They concluded that the BAPAT as a presumptive test .The superior sensitivity of the BAPAT is resultant to the low final antigen concentration (3%) after the addition of serum and final pH of the test (4.02 ± 0.04) permits greater analytical bias in favour of detection of IgG1.

Saravi et al. (1995) demonstrated that BAPAT is superior to RBPT as a screening assay. The relative sensitivity estimates of the BAPAT were the highest of any comparison of all assays being compared.

Sayour (1995) reported that the CFT was confirmed as the best definitive test, which gave maximum balance of sensitivity and specificity and correlated best with culture results and the active immune response of animals. The BAPAT proved an excellent presumptive test

Abdel-Gawad (1996) in Ismailia and port-said provinces examined 1816 cows and found that the positive reactors by using SAT, BAPAT, and RBPT were 9.3%, 10.4%, and 6.2% respectively.

Ibrahim (1996) recorded that the superior sensitivity of BAPAT is mainly attributed to the low Final antigen concentration (3%) after the addition of serum. Moreover, the final pH of the test (4.02 ± 0.04) is slightly higher than that of RBPT pH (3.65) which allows inactivation of IgM and favours the detection of IgG.

Kadery (1996) In Sharkia governorate examined 2330 cattle for brucellosis by application of BAPAT, RBPT, and TAT, The results of positive reactors were in BAPAT (0.99%), RBPT (0.94%) and TAT (0.86%) .

Sawalhy et al. (1996) reported that a correlation between BAPAT and SAT results means that the SAT may miss some infected animals especially in the chronic stage of the disease and also the superior sensitivity of BAPAT which is used recently as a screening test for detection of brucellosis among cattle.

Hamdy (1997) concluded that BAPAT is the test to start with as it is sensitive, specific, quick and easy to be done and interpreted. BAPAT has a superior sensitivity compared with other serological tests due to the final pH (4.02+0.04). This pH allows some of the IgM besides IgG1, IgG2 and IgA to share in the reaction. BAPAT had a higher sensitivity than RBPT attributed to the amount of serum used in BAPAT which is greater than that of RBPT. Besides the pH 3.65 of the Rose Bengal antigen allows less amounts of IgM to share in the reaction of RBPT

Montasser, et al (2001) concluded that the SAT, and BAPAT, were highly sensitive presumptive tests while the Rivanol test, and CFT, were specific confirmatory tests.

Torky et al .(2001): examined 4357 cattle serum samples were collected and examined for brucellosis by different serological tests including (BAPAT), (RBPT), (TAT), (Riv.T)and (CFT).The obtained results revealed that the infection was high among cattle of El-Behera followed by EL-Minia, Beni Suef, EL-Menofeia and Cairo governorates ,In contrast Kafr El-Shiekh, Dammita and Alexandria governorates gave negative results. BAPAT, gave the highest rate of positive cattle (10.63%) followed by RBPT (10.19%) and Riv.T (8.70%), CFT and SAT reacted positively with 8.49% and 6.13% respectively.

Pugh., (2002) serological testing of animals alone may lead to a false diagnosis but various agglutination, and complement fixation tests indicated to detect carriers.

Khoudiar R . M (2004) reported that BAPAT was slightly more sensitive than RPBT. The authors indicated that the sensitivity of Riv. and CF tests is lower than that of RBPT and reported that the positive reactors percentage by BAPAT and RBPT were (10.63%) and (10.19%) among cattle. These findings may be due to the fact that RBPT antigen has acidity of pH 3.65.This lower pH of RBPT antigen inhibits the activity of IgM and detects IgG (enhance the agglutination). BAPAT has pH 4 which permits the detection of IgM and IgG. This explains why BAPAT is more sensitive than RBPT.

Material

Brucella abortus antigen for BAPAT: This antigen was obtained from the Veterinary Serum and Vaccine Research Institute (VSVRI), Abbassyia, Cairo, Egypt.

It is a crystal violet and brilliant-green stained Br. abortus strain 99 cells at a concentration of 11% in lactate buffer (pH 3.7 ± 0.03). It was prepared according to the USDA method (**Alton et al., 1988 and Mehanna, 1989)**

Methods

The test was carried out according to **Alton et al. (1988).** Serum to be tested and antigen were brought at room temperature before testing. 0.08 ml of each serum sample under testing was placed as one sample in one square on a glass board. The antigen was thoroughly mixed and one drop (0.03 ml) from it was dropped for each serum sample. The serum and the antigen were mixed well using a sterile spreader.

Result: The results were recorded immediately after 4 and 8 minutes. A known control positive and negative sera were included in the test. Any reaction in the form of "flocculation" was considered as positive.

DISCUSSION

The results of The previous worker declared sensitivity and superiority of BAPAT rather than RBPT. The higher percent of reactors detected by BAPAT was similar to that recorded previously by Salem **et al., (1984); Farid et al., (1995); Khoudair (2000), Montasser et al., (2001), Torky et al., (2001) and Khoudair (2004).** The BAPAT test detected a higher number of positive as compared with other serological tests and this attributed to the final pH of a serum-antigen mixture of 4.02±0.04 **(Alton et al., 1988),** this pH enables some of the IgM, beside, IgG1, IgG2 and IgA to share in the reactions **(Wright and Nielsen., 1990).** The high sensitivity of BAPAT if compared to that of RBPT may attributed to the amount of serum used in BAPAT (0.08 ml) is greater than the amounts of serum used in the RBPT (0.03 ml). On other hand, the relatively acidic pH of the RBPT (3.65) permits lesser amounts of IgM to share in the reaction, since this class of immunoglobulin is known to be acid labile **(Allan et al., 1976**). Furtherly **OIE., (1992)** reported that buffered brucella antigen tests are suitable for screening herds and individual animals

It appeared that the BAPAT, among all serological tests used gave the highest rate of positive animals (10.92%). Similar that mentioned by **Angus and Barton (1984), Dohoo et al. (1986), Hamdy (1992) and Hosein (1996)**. The high sensitivity of this test mainly is that it detects both IgG and IgM molecules **(Nelson, 1989**). Even IgG1, which is not a good agglutinating material at neutral pH, is active at low pH of BAPAT **(Macmillan, 1990).** The BAPAT is a plate test carried out in one dilution (0.08 ml serum to 0.03 ml antigen) a method which renders the test highly sensitive. This agree with **Nicoletti and Muraschi (1966)** who reported that BAPAT is more sensitive than CFT and Riv.T.

El-Gibaly et al. (1990) who concluded that BAPAT is the most sensitive test for diagnosis of Br. melitensis infected cows. **Refai (1989)** reported that it was

decided to use the buffered acidified plate antigen test (BAPAT) as a presumptive due to its high sensitivity. Positive samples should be then tested by Tube agglutination (TAT) and Rose Bengal (card) test. For confirmation, Rivanol test and if possible CFT may be used.

It was seemed from that BAPAT test gave a high percentage of reactors due to its high sensitivity than other tests. However it is considered that this test is not conclusive and should be supported by one or more other serological tests.

When we tested the same samples using Rose Bengal Plate Antigen test (RBPT), This test does not show a great difference with the results of BAPAT which means that the test is less sensitive and once again should be supported by other serological tests. This agree with those obtained **by Angus and Barton (1984), Hamdy (1992) and Anwar (1999).**

It may be worthy to note that, on infection, IgM appears earlier than other immunoglobulin **(Morgan et al.,1978 and Alton et al.,1988).** This would also explain the higher percentage of positive cases detected by RBPT and points out the fact that, this test could be of help in detecting cases of recent infection which not diagnosed by the SAT. Moreover an advantage reported for this test is that , the acidic pH of the Rose Bengal test (3.65) may inhibit the non-specific antibodies leaving the specific agglutinins to react in the test **(Corbel, 1973; Patterson et al., 1976).** As a result of this oversensitivity, the test is best applied as an initial screening test during an eradication programmes. Sera reacting to it should be subjected to other confirmatory tests **(Nicoletti 1967).**

Nicoletti (1969) reported that data indicated that serum agglutination test (SAT) detected only 52% of the cultured positive cattle in infected herds, while buffered acidified plate antigen test (BAPAT) detected 95%, Rivanol test (Riv.T) detected 96% and complement fixation test (CFT) detected 98%. The (SAT) was the most accurate in detecting 83% of cattle as non infected, whereas the supplemental tests such as Riv.T and CFT correctly detected 73% and 62.9% respectively as non infected

Methods Used for Handling and Preservation of Blood Samples and Tissues for Brucella Examination

Methods Used for Handling and Preservation of Blood Samples and Tissues for Brucella Examination

By

Dr. Abdel-khalik M. Montasser

I Sampling

1: Type of samples from live animals

- Blood
- Milk
- Foetal fluids
- Swaps (vaginal)
- Semen
- Cheese

2: Type of samples from slaughtered animals:

- Lymph nodes (Retro pharyngeal Supra mammary internal iliac)
- Tissue from (spleen and liver)
- Organs (ovaries testis)

3: Type of sample from aborted animals:

- Stomach content of aborted fetus
- Tissue of aborted fetus (lung spleen)
- Vaginal swab from dam if present.

II- Sampling sheets must be as follow:

Confirmatory tests Sheet for Brucella

Governorate : ***Districts:***

Village/Farm: State:

Total tested animals: Lab code number: Date: / /

Serial No.	An. No.	Animal species	Age	Sex	Serological Result					Conclusion
					TAT	BAPAT	RBT	CFT	Riv.T	
1.										
2.										
3.										
4.										
5.										
6.										
7.										
8.										
9.										
10.										

Vaccine Brucella Test Sheet

Governorate: *Districts:*

Village/Farm: **State:**

Total tested animals: *Lab code number:* *Date: /*

Serial No.	An. Sp.	An. No.	Age	Sex	Type of vaccine	Serological .Result Befor vaccination	Remark
1							
2							
3							
4							
5							
6							
7							
8							
9							
10							

III .How to collect samples

1:Blood samples

Blood samples were collected from animals, About 10 ml of whole blood were obtained through jugular vein puncture of cattle, sheep, goats, and camels in sterile test tubes or vacutainer. The blood samples were kept in a slant position about two hours far away from sunlight, and then transferred to the laboratory. The blood samples were kept overnight at 4 °C to allow separation of serum then centrifuged at 3000 rpm for 10 minutes. The clear serum was transferred into two test tubes for each sample and labeled. One tube was used immediately and the other was stored in the deep freezer at -20 °C for further investigation.

2: Milk sample:

In sterilized McCartney the milk is collected from each animals. The whole udder is washed and dried and the end of the teat is disinfected with a swap of alcohol and wiped dry the teat in sides away from the operator being treated first. The samples of milk are taken first from the teats nearest the operators before proceeding to those on the off side.

The first one or two streams of milk are discarded and then a sample from each teat is milked directly into a sterile bottle. It is essential to avoid contact between the stream of milk and milkers hands. The hand should be washed in mild disinfectant between each animal.

3: Vaginal swaps:

Samples may be taken by vaginal or preputial washing, or by the use of sutable sSometimes the servix or urethra are also sampled by swabbing. wabs. Cotton swaps are protected in a glass tube about 8 mm in internal diameter and about 10 cm long the wall is enclosed and sterilized in a hot air oven. The swap is taken from the test tube and introduced into animal vagina. The swap is then pushed forward out of the tube and rotated genteelly in several directions before being pulled back into the tube.

4: Aborted fetus:

The most valuable materials from aborted fetus for cultural examination are the stomach contents, lung and spleen, which must be taken with a septic precautions to sterile container.

5: Semen:

Samples of semen are best obtained using an artificial vagina or by artificial stimulation.

TRANSPORT OF SAMPLES

Samples (referred to as Diagnostic Specimens in official regulations) must be carefully packed, to avoid any possibility of leakage or cross-contamination. They should be delivered within 48 hours and should usually be kept cool during transit.

Some samples should not be frozen. Screw-capped bottles should be used and should be additionally sealed with adhesive tape or paraffin wax.

Samples in individually identified containers should be placed in larger strong outer containers and packed with enough absorbent material to protect from damage.

Serum samples can give a lot of information about the animals from which the sera were taken. There are many different tests that can be done on sera, to assay a variety of constituents, such as immunoglobulins.

When a sufficient number of serum samples have been collected at random from a population, valid comparisons can be made of sex, age, breed and geographical location.

High risk groups can be identified, vaccination priorities established, and patterns and rates of disease determined

Rose Bengal Plate Test

Rose Benegal Plate Test

Dr. Hazzem Mohamed Mahmoud

Rose bengal plate test was done according to Morgan et. al.,(1969)

- **Principle:**

It is a qualitative plate agglutination test that uses a colored acidified antigen (pH 3.65, cell volume 8%) to inhibit non-specific reactions due to IgM and enhance the agglutination ability of specific IgG1.

Use:

It is a quick easy screening test that minimizes the number of samples requiring further serological confirmation.

Antibody selectivity: RBPT

Antibody	IgM	IgG_1	IgG_2	IgA
Detection	±	+++	+	±

Procerure of the Rose Bengal Plate test

* The samples and antigen were first brought to room temperature.

* Using an adjustable micropipette, 30µl. of the sample were placed on the centre of one tear- drop area of the brewer diagnostic card.

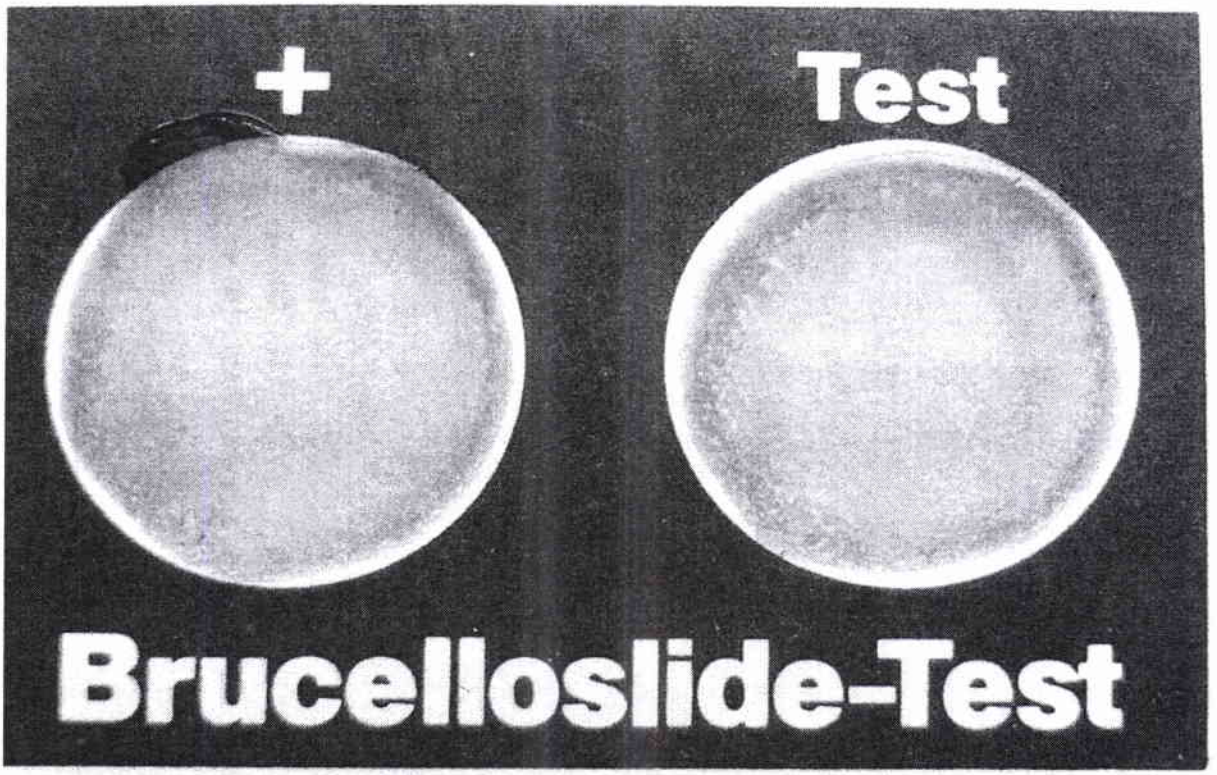
Control positive and negative samples were included.

* Thirty µl of antigen were dispensed adjacent to the sample, mixed using the stirrer and spread over the entire "tear-drop" test area.

* After mixing, cards were slowly rocked using the brucellosis-cardrocker producing 12 to-and-frotilting motions per minute for 4 minutes.

Interpretation of the RBPA test

As the test is done at one dilution, any degree of agglutination within minutes (≥25IU/ml) was considered positive. No agglutination within 4 minutes was regarded as negative.



Milk ring test (MRT)

In lactating animals, the MRT can be used for screening herds for brucellosis. In large herds (>100 lactating cows), the sensitivity of the test becomes less reliable. False-positive reactions may occur in cattle vaccinated less than 4 months prior to testing, in samples containing abnormal milk (such as colostrum) or in cases of mastitis. Therefore, it is not recommended to use this test in very small farms where these problems have a greater impact on the test results.

Antigen production:

MRT antigen is prepared from concentrated, killed *B. abortus* S99 or S1119-3 cell suspension, grown as described previously. It is centrifuged at, for example, 23,000 *g* for 10 minutes at 4°C, followed by resuspension in haematoxylin-staining solution. Various satisfactory methods are in use; one example is as follows: 100 ml of 4% (w/v) haematoxylin (Cl No. 75290) dissolved in 95% ethanol is added to a solution of ammonium aluminium sulphate (5 g) in 100 ml of distilled water and 48 ml of glycerol. 2 ml of freshly prepared 10% (w/v) sodium iodate is added to

the solution. After standing for 30 minutes at room temperature, the deep purple solution is added to 940 ml of 10% (w/v) ammonium aluminium sulphate in distilled water. The pH of this mixture is adjusted to 3.1, and the solution must be aged by storage at room temperature in the dark for 45–90 days.

Before use, the staining solution is shaken and filtered through cotton wool. The packed cells are suspended in the staining solution at the rate of 1 g per 30 ml stain, and held at room temperature for 48 hours (some laboratories prefer to heat at 80°C for 10 minutes instead). The stained cells are then deposited by centrifugation, and washed three times in a solution of sodium chloride (6.4 g), 85% lactic acid (1.5 ml) and 10% sodium hydroxide (4.4 ml) in 1.6 litres of distilled water, final pH 3.0. The washed cells are resuspended at the rate of 1 g in 27 ml of a diluent consisting of 0.5% phenol saline, adjusted to pH 4.0 by the addition of 0.1 M citric acid (approximately 2.5 ml) and 0.5 M disodium hydrogen phosphate (approximately 1 ml) and maintained at 4°C for 24 hours. The mixture is filtered through cotton wool, the pH is checked, and the PCV is determined and adjusted to approximately 4%.

Test procedure :

The test is performed on bulk tank milk samples. If necessary, samples could be pretreated with preservative (0.1% formalin or 0.02% bronopol) for 2–3 days at 4°C prior to use. The test is performed by adding 30–50 µl of antigen to a 1–2 ml volume of whole milk (the volume of milk may be increased for bulk samples from larger herds). The height of the milk column in the tube must be at least 25 mm. The milk samples must not have been frozen, heated, subjected to violent shaking or stored for more that 72 hours. The milk/antigen mixtures are normally incubated at 37°C for 1 hour, together with positive and negative working standards. However, overnight incubation at 4°C increases the sensitivity of the test and allows for easier reading. A strongly positive reaction is indicated by formation of a dark blue ring above a white milk column. Any blue layer at the interface of milk and cream should be considered to be positive as it might be significant, especially in large herds. The test is considered to be negative if the colour of the underlying milk exceeds that of the cream layer.

Antibody selectivity: MRT

Antibody	IgM	IgG_1	IgG_2	IgA
Detection	++	++	++	++++

الاختبار الشريحي المخمد المحمض :

- **Buffered a acidified plate antigen test (BAPA)**

هو اختبار شريحي نوعي وليس كمي يستخدم فيه أنتجن مخمد محمض ملون ذو أس هيدروجيني (3.8) (Ph 3.8 ± 0.05) يمكنه الكشف عن الأجسام المناعية من النوع IgM , gG_1 Ig G_2, بدرجات متفاوتة

- وهو اختبار ذو حساسية عالية .

الأدوات المستعملة في الإختبار :

لوح زجاجي – ماصة أتوماتيك مع الأقماع الخاصة بها – أعواد تقليب – انتجن مخمد محمض – قطارة تعطي نقطة معيارية مقدرها 30 ميكرون .

طريقة عمل الاختبار :

1- تجهيز العينات والأنتجن وذلك بإخراجهم من الثلاجة قبل إجراء الاختبار بمدة 30 – 60 دقيقة حتى يأخذ كلاً منهما درجة حرارة الغرفة .

2- بواسطة الماصة الأتوماتيك تؤخذ كمية مقدارها 80 ميكرون من السيرم وتوضع على اللوح الزجاجي .

3- توضع نقطة من الأنتجن مقدارها 30 ميكرون بواسطة القطارة المخصصة لذلك .

4- يقلب الأنتجن مع السيرم بواسطة أعواد التقليب ويترك لمدة 8 دقائق ثم تقرأ النتائج.

أ) إيجابي : وجود تلازن (تجمع) بين السيرم والأنتجن .

ب) سلبي : يبقى الخليط كما هو متجانس ولا يوجد لأثر تلازن .

- **اختبـــــار اللبـــن الحلقـي Milk Ring test**

اختبار سريع كمي يستخدم انتجن ملون يمكنه الكشف عن جميع أنواع الأجسام المناعية في اللبن ذو حساسية عالية .

- **الأدوات المطلوبـــــــة :**

* حوامل أنابيب (راكات) أنابيب ويزرمان :
* أنتجـــــن اللبـــن الخلقـــــي .

- **طريقـــة إجراء الاختبار :**

1- ترج عينة اللبن جيداً عدة مرات .

2- يوضع مقدار 1سم من اللبن في أنبوبة ويزرمان .

3- توضع نقطة من الأنتجن (30 ميكرون) .

4- يقلب الانتجن واللبن جيداً بقلب الأنبوبة عدة مرات .

5- تترك الأنابيب الحضانة لمدة (ساعة) ثم تقرأ النتائج .

- **قـــــــراءة النتائـــــــج :**

أ- حلقة اللبن بيضاء وعمود اللبن أزرق (سالبة –)

ب- حلقة اللبن ملونة بزرقة خفية وعمود اللبن أزرق (+)

ج- حلقة اللبن بلون أزرق نفس درجة عمود اللبن (+ +)

د- حلقة اللبن بلون أزرق أكثر عمقاً من عمود اللبن (+ + +)

هـ- حلقة اللبن زرقاء تماماً وعمود اللبن أبيض تماماً (+ + + +)

اعتبـــــاراً من (+ +) فأكثر تعتبر العينة إيجابيـــة .

- **مميـــــــزات الاختيـــــــــار .**
  - حساسية عامة .
  - سريـــــــــع .
  - يمكن الكشف عن جميع أنواع الأجسام المناعية .
  - يمكـــن إجـــراءه علـــى اللـــبن الموجـــود بالتانكـــات حيـــث أن تخفيـــف اللـــبن لا يـــؤثر بدرجـــة كبيرة على حساسية لاختبار .

- **عيـــــــوبـــــــــه :**
  - لا يمكن إجراءه إلا على الحيوانات الحلابة .
  - لا يمكن إجراءه على الحيوانات الجافة ، والعجلات والذكور .
  - الحيوانات المصابة بالتهاب الضرع تعطي نتائج كاذبة .
  - يعطي نتائج خاطئة في بداية فترة الحليب ونهاية فترة الحليب .

- **اختيـــــار الروزبنجـــال** **Rose Bengal plate test (RBPT)**

اختيار شريحي سريع نوعي وليس كمي شريحي يستخدم فيه انتجن حامض ذو أس هيـــدروجيني 3.65 (Ph 3.65 ± 0.05) بهدف تثبيط الأجسام المناعية من النوع IgM والتي تتسبب في النتائج الإيجابية الكاذبة

- **الأدوات المستعملـــــة فـــــي الاختبـــار :**

لوح من الملامين الأبـــيض – ماصـــة أتوماتيـــك مـــع الأقمـــاع الخاصـــة بهـــا – أعـــواد تقليـــب – انتجن روزبنجال الملون – قطارة تعطي نقطة معيارية مقدرها 30 ميكرون .

- **طريقـــــة عمـــــل الاختبـــــار ..**

1- تجهيـــز العينـــات والأنـــتجن وذلـــك بـــإخراجهم مـــن الثلاجـــة قبـــل إجـــراء الاختبـــار بمـــدة 30 – 60 دقيقة حتى يأخذ كلاً منهما درجة حرارة الغرفة .

2- بواســـطة الماصـــة الأتوماتيـــك تؤخـــذ كميـــة مقـــدارها 30 ميكـــرون مـــن الســـيرم وتوضـــع على لوح من الميلامين الأبيض .

3- توضع نقطة من الأنتجن مقدارها 30 ميكرون بواسطة القطارة المخصصة لذلك .

4- يقلب الأنتجن مع السيرم بواسطة أعواد التقليب ويترك لمدة 4 دقائق ثم تقرأ النتائج.

أ) إيجابي : وجود تلازن (تجمع) بيـــن السيرم والأنتجن .

ب) سلبي : يبقى الخليط كما هو متجانس ولا يوجد لأثر تلازن .

Buffered acidified plate antigen test (BAPA test)

- Principle:
- It is a qualitative plate agglutination test that uses a colored acidified antigen (pH 3.8 ± 0.05) to inhibit non-specific reactions due to IgM and enhance the agglutination ability of specific IgG1.
- Use:
- It is a quick easy presumptive test to start with in order to exclude negative samples from further serological tests
- Antibody selectivity: BAPA

Antibody	IgM	IgG_1	IgG_2	IgA
Detection	+	+++	++	±

Procedure of Buffered acidified plate antigen test(Anon, 1984);

*The samples and antigen were allowed to come to room temperature

*Eighty µl of sample were placed on the center of a square-etched area of the double-strenght glass plate of Minnesota testing box.

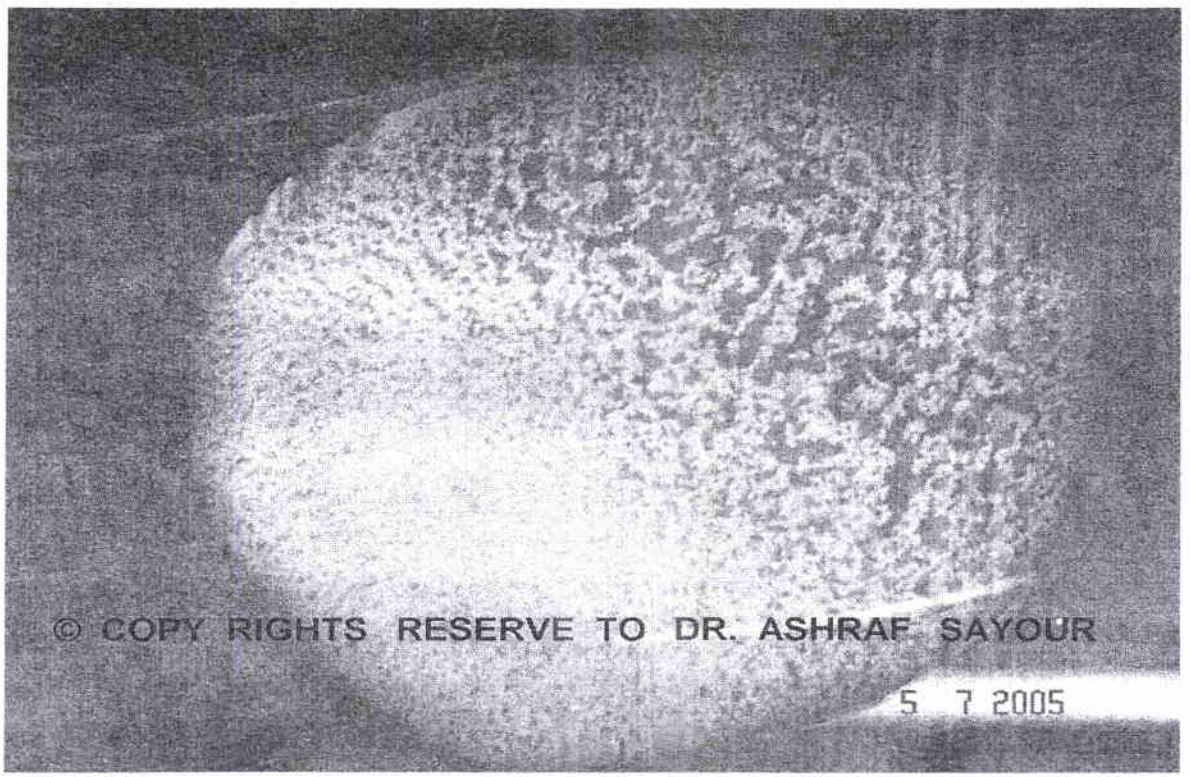
*Thirty lµ of antigen were dropped adjacent to the sample on to the center of each square-etched area. The sample and antigen were thoroughly mixed

*The plate was rotated 4 times, left for 4 minutes in the Minnesota box with the lead covered to prevent evaporation, rotated 4 times agaain, incubated for another 4 minutes in the box and finally rotated 4 further rotations.

*The plate was examined for agglutination while wet with the light of Minnesota box turned on .

Interpretation of the BAPA

Any degree of agglutination within 8 minutes was considered positive (≥20 IU Ab/ml).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The Complement Fixation Test for the Diagnosis of Brucellosis

The Complement Fixation Test for the Diagnosis of Brucellosis

By

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The CFT was used along with the standard tube agglutination test (SAT) in the earliest investigations on serodiagnosis of bovine brucellosis in Denmark by **Holth (1909)** and in England by **McFadyean and Stockman (1909). Hadley and Beach (1912)** and **Larsen (1912)** began preliminary use of the CFT in the United States. Despite the statements of some authors **(Carpenter and Boak, 1930; Wise and Craig, 1942)** that CFT did not offer advantage over the agglutination test, and that the sera were often anti-complementary, CFT had been widely used in the USSR as early as 1938 **(Hoptman, 1959)** and in some European and South American countries for the diagnosis of brucellosis in farm animals.

Observations that the test was a better indicator of infection than the SAT and that the reactions to the CFT receded sooner than those to the SAT test after S19 calfhood vaccination, together with technical improvements in reagents and apparatus, increased interest in the test.

Principle (2 main steps)

1. **Fixation of complement to the antigen-antibody complex:** Diluted antigen and the serum under test (deprived of its complement by heat inactivation) are incubated in the presence of normal guinea pig serum, which provides a source of complement (Normal guinea pig serum contains a greater concentration of all complement components than other serums like those of cattle, horses, sheep, …etc., i.e. it lyses RBCs well).
2. **Addition of a tracer to visualize the 1st step, if any:** The amount of free complement remaining is measured by adding an indicator system consisting of antibody-coated sheep RBCs. Sheep RBCs are characterized by being more fragile than those of other animal species.

Titration of CFT ingredients

Detailed account on the titrations of complement, haemolysin and antigen as well as preparation of CFT reagents are available elsewhere in **Alton *et al.* (1988)**, **MacMillan (1990)** and **OIE (2004)**.

CFT proper test (warm microtechnique)

1. Sera under test are inactivated in water bath at 58° C for 30 minutes on the day of testing.
2. Using a 5-50 µl. adjustable 8-channel micropipette, 25 µl. of diluent are added to all wells in separate plates.
3. Doubling dilutions of heat-inactivated sera are made in a row-wise manner producing dilutions up to 1/4096. Similarly, 25 µl. of strong positive, weak positive and negative control sera are included.
4. Twenty five µl. of freshly diluted antigen are added starting at the second wells (the first well being a serum anti-complementary control). Twenty five µl. of diluent are added to these wells in lieu of antigen.
5. Twenty five µl. of five 50% haemolytic units of complement (5 $C'H_{50}$) are added to each well. Plates are covered and placed at 37° C for 30 minutes with the complement controls prepared in macro-volumes as follows.
6. Fifty µl. of the working dilution of complement and 0.7 ml. of diluent are added to each of 4 tubes and placed at 37° C for 30 minutes. All tubes receive 0.25 ml. of sensitized RBCs and are placed in the water bath at 37° C for 30 minutes, shaking from time to time. One ml. of cold diluent is added to each tube and the tubes centrifuged. ODs of the supernatents are determined spectrophotometrically, amalgamating pairs of tubes to provide adequate volume for the spectrophotometer. The percents of haemolysis are calculated as before. Figures between 25-50% are acceptable.
7. Twenty five µl. of sensitized RBCs are added to each well. Plates are covered and placed in the shaker-incubator at 37° C for 30 minutes, then placed at 4° C to allow for the unlysed RBCs to settle down.
8. Plates are read from below using a viewer with magnifying mirror starting from the 1/4 serum dilution.

Reading of results and end point

Complete fixation (no haemolysis) recorded as ++++ or 100% fixation.

Complete lack of fixation (complete haemolysis) recorded as 0% fixation.

Degrees of partial haemolysis are determined as +++ (75% fixation), ++ (50% fixation or haemolysis) or + (25% fixation).

The end titre is the highest serum dilution with 25% fixation or greater.

Standardization of the CFT

Mac Millan (1990) reported that the European Economic Community (EEC) has adopted the German Standard for anti-*Br. abortus* serum as the EEC Standard for CFT; it has been calibrated against the ISAbS. Its CFT activity was defined as 1000 EEC CF units per ampoule. Results of an international collaborative assay to compare the CF activity of the First, Second and EEC Standards showed that the EEC CF unit was equivalent to the ICFTU. The EEC considers any serum containing 20 EEC CF units/ ml (one-fiftieth of the ISAbS activity) or more as positive.

Calculation of the ICFTU

The second ISAbS containing 1000 ICFTU.ml.$^{-1}$, was used to establish a conversion factor for converting CFT titres into ICFTU.

Conversion factor = 1000/ CFT titre of the ISAbS

Conversion factor (for 1+ complement fixation) = 1000/ 320 = 3.125

The lowest limit of detection

6.25 ICFTU.ml^{-1}.

Minimum diagnostic levels

Positive: ≥ 20 ICFTU.ml^{-1}.

Suspect: 10 ≤ ICFTU.ml-1< 20.

Antibody isotype selectivity

IgM (±), **IgG$_1$** (+++), **IgG$_2$** (-), **IgA** (-)

Relative sensitivity

The CFT is generally more sensitive than the SAT especially in recent and chronic longstanding infections. It is slightly more sensitive than the MET, Riv.T but not the indirect and the competitive ELISA.

Relative specificity

The test is generally more specific than the SAT, MET, Riv.T and indirect ELISA, but not the competitive ELISA.

Diagnostic value and use

The test is widely used in eradication programs and has reduced the time required for eradication and the number of overcondemnations. In sheep and goats, the CFT is considered to be the most specific and sensitive method for the serological diagnosis of *Br. melitensis* infection. Being a standard quantitative test, this makes CFT a reliable test for international trade of animals as recommended by the **OIE (2004)**.

Alton *et al.* (1975) found that all culturally positive animals reacted to the CFT. **Nicoletti (1977)** reported that the CFT correctly identified 98% of culturally positive animals, and **Berman (1977)** isolated *Brucella* from 134 of 303 animals with CFt titres of 1/40 or more, but from only 1 of 82 cows with CFT titres of 1/20 and from none with titres below 1/20. Some culturally positive animals fail to show a positive CFT reaction, especially at the time of abortion. This phenomenon can be found for most of the serological tests.

Advantages

- High accuracy (precision)
- A variety of antigens can be used for the CFT

Disadvantages

- The technique is time, effort and proficiency demanding
- The problem of anticomplementary sera
- The problem of prozones
- The problem of haemagglutination

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التشخيص التفريقي بين البروسيلا والأمراض الطفيلية المسببة للإجهاض

التشخيص التفريقي بين البروسيلا والأمراض الطفيلية المسببة للإجهاض

إعداد

أ.د / سهير محمد المنياوى

رئيس قسم بحوث الطفيليات

معهد بحوث صحة الحيوان

الإجهاض هو عبارة عن خروج الجنين حياً من الرحم قبل أن يكتمل نموه أو ميتاً في أي مرحلة من مراحل الحمل .

ويعتبر حدوث الإجهاض في أي قطيع من حيوانات المزرعة (الأبقار والجاموس والجمال والأغنام والماعز) مشكلة اقتصادية كبيرة ولها أسباب كثيرة ممكن أن تكون فيروسية أو بكتيرية أو فطرية أو طفيلية .

وتعتبر الطفيليات أحد هذه الأسباب الرئيسية للإجهاض وهذا يؤثر سلبيا على مجمل إنتاجية المزرعة واقتصادياتها

وتتهم بعض الطفيليات دون الأخرى في إحداث المرض ومن الطفيليات التي تساهم في جزء كبير من هذه الحالات هي الطفيليات الأولية " Protozoa " وهي طفيليات وحيدة الخلية إما أن تكون في الدم أو في الأنسجة .

طفيليات الدم :

وهى عبارة عن طفيليات أولية توجد داخل كرات الدم الحمراء مثل

Babesia , Theileria & Anaplasma.

والنوعان الأول والثاني هي التي تسبب الإجهاض للأبقار والجاموس والبعض الآخر من هذه الطفيليات يكون خارج كرات الدم الحمراء مثل Trypanosoma, Microfilaria

طفيليات الأنسجة :

يمكن أن تحدث الطفيليات الأولية التي توجد داخل الأنسجة الإصابة بالإجهاض على مدار العام وأن تصيب بعض من أو كل القطيع ، وهناك أيضا بعض الحيوانات مثل الأغنام والماعز تكون أكثر عرضة للإجهاض عن الحيوانات الأخرى نتيجة الإصابة بطفيل التكسوبلازما Toxoplasma .

وهناك أيضا طفيل النيوسبورا Neospora حيث يعتبر السبب الرئيسي للإجهاض في الأبقار الحلابة .

وأيضا طفيل الساركوسيست Sarcocysts الذي يحدث الإجهاض في إناث الأبقار والأغنام ويسبب النفوق في الحملان الصغيرة .

طفيليات الدم

1 - طفيل البابيزيا :

- طفيل أولى يعيش داخل كرات الدم الحمراء .
- شكل الطفيل أما أن يكون كمثريا أو بيضاويا أو أميبيا أو كرويا
- حجم الطفيل يترواح من 1.5 الى 5 ميكرون .
- يصيب كل المجترات والخيول والكلاب .
- يسبب داء البابيزيا Babesiosis Red water Fever .
- الطفيل ينقسم فقط داخل كرات الدم الحمراء انقساما لا تزواجيا .
- العائل الناقل له وهو القراد الصلب Hard Ticks .
- يكون النقل مباشر أو عن طريق العبور من مبيض أنثى القراد المصابة .
- من أهم الأعراض
  1. الحمى Fever
  2. أحمراء البول Haemaglobinuria .
  3. اضطرابات هضمية
  4. إجهاض
  5. ضعف عام نقص في وزن وإنتاج الحيوان خاصة الأبقار ر المدرة للبن

2- طفيل الثيليريا :

- يعتبر طفيل الثيليريا من أصغر الأوليات حيث يتراوح حجمه من 1-2 ميكرون .
- وهو عبارة عن طفيل مستدير أو بيضاوي أو عصوي أو كمثرى الشكل أو غير منتظم الشكل .
- يصيب المجترات (الأبقار – الجاموس – الجمال – الماعز – الأغنام) .
- وهذا الطفيل ينقسم داخل الخلايا البلغمية Lymphocystes ويحدث الانشطار داخل الخلايا معطية أقسومات وهذه تتجمع بشكل أجسام مستديرة أو غير منتظمة الشكل تبلغ حجمها 2-12ميكرون وإذا لونت بصبغة جيمسا فإنها تأخذ اللون الأزرق ذات نويات حمراء ولذلك تعرف باسم أجسام كوخ الزرقاء Kock´ s blue bodies
- أما الأشكال التي داخل كرات الدم الحمراء فلا تستطيع الانقسام .
- العائل الناقل هو القراد ا لصلب من نوع ثنائي أو ثلاثي العائل .
- ويكون النقل بطريقة من طور إلى طور Transstadial transmission .

- ويسبب مرض ثيلاريوزا البقر Bovine Theileriosis المعروف بمرض حمى الساحل الشرقي East Coast fever
- ومن الأعراض المميزة لثيليريا

1. ارتفاع درجة الحرارة 41-42 م
2. تضخم فى الغدد الليفماوية السطحية
3. الإجهاض
4. دموع غزيرة
5. الانيميا وضعف المقاومة قلة ادار اللبن ونقص الوزن .

وللتفرقة بين البروسيلا وهذه الأمراض الطفيلية المسببة للإجهاض

فلابد من تشخيص الحالة فهو مهم وتحديد الطفيل المسبب للمرض غاية فى الأهمية .

ويعتمد الاشتباه في الإصابة بطفيليات الدم في الحيوانات المصابة على صورة الأعراض المرضية مع الاهتمام بتميز الاختلافات فى الأعراض بين الإصابات بطفيليات الدم المختلفة فمثلا نلاحظ أن **حالات الثيليريا** تكون مميزة بتضخم الغدد الليمفاوية الظاهرة وعدم وجود بول غامق

أما **حالات البابيزيا** تكون مصحوبة بالبول الغامق والأنيميا وعدم وجود تضخم بالغدد الليمفاوية .

ويعتبر وجود القراد من القرائن التي تقوى الاشتباه في الإصابة بطفيليات الدم .

وأما التشخيص المؤكد فيعتمد على التشخيص الميكروسكوبي للطفيل في الدم حيث يمكن التعرف على الطفيل في :

أ – شرائح الدم المصبوغة

ب – مسحة من غدة ليمفاوية ظاهرة متضخمة .

ويفضل لطفيليات الدم استخدام صبغة جيمسا Giemsa Stain .

أ – شرائح الدم المصبوغة :

عمل شريحة الدم :

أساس نجاح عمل شريحة الدم هو

- تنظيف الشرائح الزجاجية بغمسها في الكحول .
- ثم تجفف بقطعة قماش نظيفة جافة خالية من الوبر أو قطعة شاش نظيفة .
- مراعاة عدم لمس سطح الشريحة بالأصابع حتى لا يترك على الشريحة أثر .
- تحفظ الشرائح نظيفة في علبة الشرائح لحين استخدامها .

تجميع عينات الدم

عند عمل شرائح من الدم تؤخذ عينة (2-3 قطرات من الدم) على الشرائح الزجاجية مباشرة من وريد الأذن بعد تنظيف الجلد من الأتربة وتطهير المكان بكحول إيثلى 70% وذلك لعمل مسحات دم رقيقة .

عمل مسحات الدم الرقيقة Thin blood film

ومن خلال المسحات الرقيقة يمكن التعرف على الطفيل .

طريقة عمل المسحات الرقيقة :

1. وضع قطرة دم قرب أحد أطراف الشريحة وتلمس قطرة الدم بنهاية شريحة أخرى أو غطاء شريحة مع زواية وضع 30درجة وتسحب شريحة الفرد للأمام
2. تترك الشريحة حتى تجف في الهواء أو باستخدام مروحة أو حضانة .
3. وعلى الجزء السميك من الشريحة وبواسطة قلم رصاص أو قلم الماظ يكتب الرقم الكودي ليسهل التعرف على الحيوان المأخوذ منة العينة .
4. يتم تثبت الشريحة " مسحات الدم الرقيقة " بكحول مثيلى نقى Absolute Methyle Alcohole لمدة 3-5 دقائق أو يترك حتى يتطاير الكحول .
5. الشريحة الجيدة الفرد تكون سميكة من ناحية ورقيقة من الناحية الأخرى

طريقة صبغ شريحة الدم بصبغة جيمسا

- بعد تثبت الشريحة بالكحول .
- يتم تخفيف الصبغة بنسبة 1: 30 (1سم صبغة ألي 30 سم ماء مقطر) .
- توضع الشريحة داخل حوض الصبغة المخففة بشرط أن تكون مغطاة تماما بالصبغة وتترك لمدة 30 دقيقة حسب تركيز الصبغة .
- ثم تغسل الشرائح تحت تيار مائي لإزالة رواسب الصبغة .
- تترك لتجف في الهواء .
- تفحص مجهريا × 100 وممكن أن نجد داخل كرات الدم الحمراء أما طفيل البابيزيا أو الثيليريا .

ب – مسحات من الغدداللليمفية الظاهرة المتضخمة :

- ولعمل مسحات أو أخذ عينات من العقد الليمفية المتضخمة نتبع الآتي :
- يطهر مكان الجلد حول العقد الليمفية بعد تنظيفه من الأتربة .
- ثم يضغط باليد حول العقدة حتى تبرز العقدة للأمام .
- ندخل إبرة حقنة معقمة في العقدة ولا نحركها حتى لا تتسبب في تهتك أنسجة العقدة0
- نضغط ضغطا خفيفا على العقدة ثم نسحب الإبرة ونركبها في السرنجة .
- ندفع السائل الموجود في الإبرة على شريحة زجاجية .
- تعامل مثل مسحات الدم الرقيقة (تجف – تثبت بالكحول – تصبغ بالجيمسا) .
- تفحص مجهريا للبحث عن أجسام كوخ الزرقاء داخل سيتوبلازم الخلايا البلغمية .

في حالة الحيوان النافق

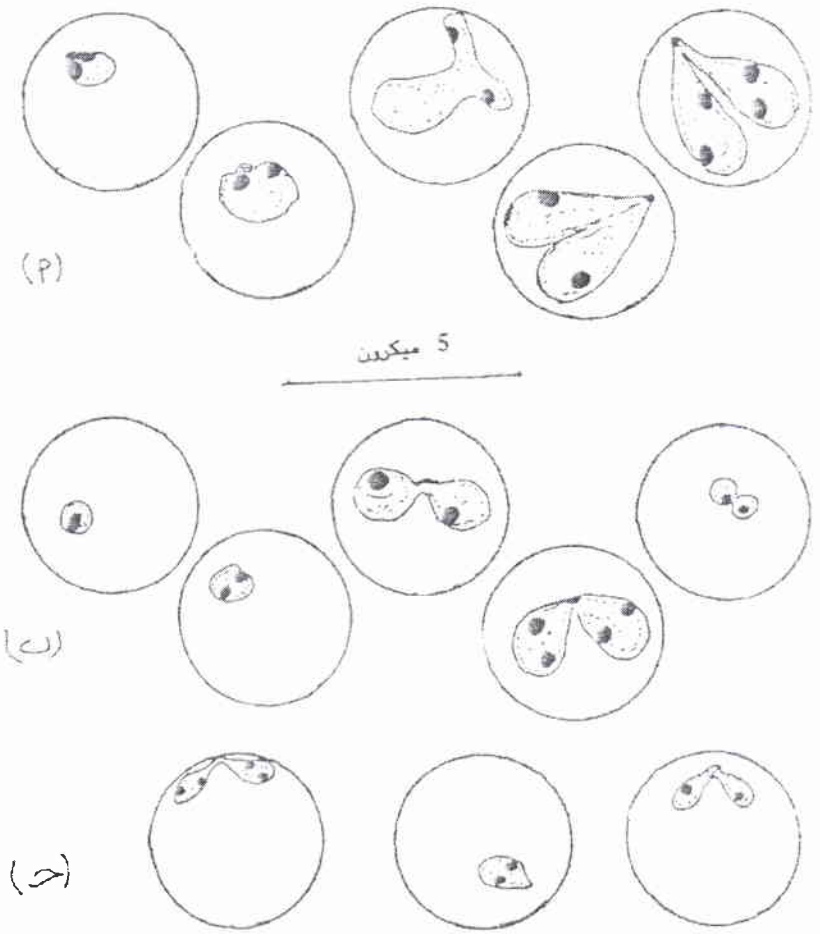
يتم أيضا أخذ مسحات دم ومسحات من الأنسجة

مسحات الدم :

أثناء إجراء الصفة التشريحية تؤخذ مسحات الدم من تجويف القلب حيث يوجد دم غير متجلط .

مسحات من الانسجة Impersion Smears

- يمكن أخذ مسحات من الأنسجة والأعضاء الداخلية مثل الكبد والطحال والعقد الليمفاوية والمخ .
- وذلك بقطع جزء من هذه الأعضاء وملامسة السطح المقطوع للشريحة الزجاجية .
- وتترك الشريحة حتى تجف في الهواء .
- وتعامل مثل مسحات الدم الرقيقة وتصبغ بصبغة جيمسا

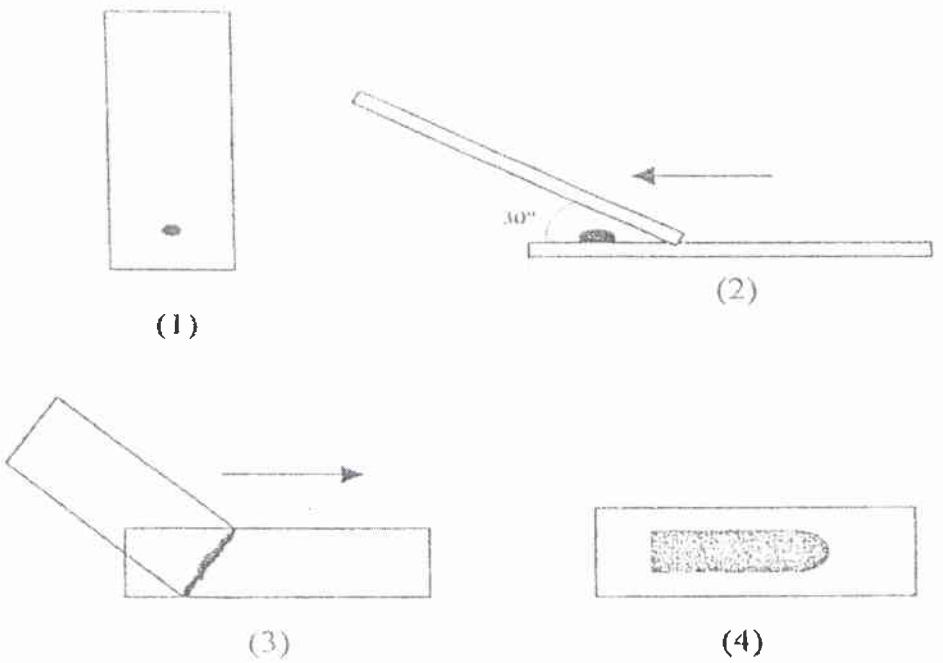


أنواع طفيليات بابيزيا (Babesia species) في الأبقار

(أ) بابيزيا (بيروبلازما) بيجمينا *Babesia (Piroplasma) bigemina*

(ب) بابيزيا (بابيزيا) بوفز *Babesia (Babesia) bovis*

(جـ) بابيزيا (بابيزيا) دايفرجنز *Babesia (Babesia) divergence*



Preparation of thin blood film

أسماء المشاركين في الدورة

قائمة المشاركين في الدورة التدريبية في مجال " التطبيقات العملية للتقنيات المتقدمة في تشخيص وإستقصاء مرض الإجهاض المعدي"

في الفترة 2006/6/15-6/11

م	اسم المشارك	الوظيفة	البلد	العنوان الإلكتروني	العنوان
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29	د. أميمة أحمد جمال الدين	طبيب بيطربي	السودان		وزارة الثروة الحيوانية والسمكية الإدارة العامة لصحة الحيوان ومكافحة الأوبئة
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رقم الإيداع: 2006/678