TRIAL FOR IDENTIFICATION OF THE DIFFERENT STRAINS OF BRUCELLA COMPARED WITH OTHER BACTERIAL SPECIES USING THE QUANTITATIVE CYTOCHEMICAL DETERMINATION OF SOME MITOCHONDRIAL ENZYMES

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ABSTRACT

There were several methods for identification of bacterial species and strains such as biochemical, serological, morphological (stains), polymerase chain reaction and others methods, We try from the current study to evaluate the quantitative and qualitative histochemical (cytochemical) techniques adapted for bacterial suspensions in order to quantitative (spectrophotometric) determination of some bacterial mitochondrial equivalent enzymes of five species and one reference strains of bacteria under all fixed factors for the two studied bacterial mitochondrial enzymes, Succinic dehydrogenase (SDH-ase) and Adenosine triphosphatase (ATP-ase). It could be obtained six significantly different optical densities (O.D.) for each of the two enzyme reactions for the following isolated bacterial strains: E.coli, Brucella melitensis (serovar: 3.field strain), Plesiomonas shigelloids, Salmonella dublin, Brucella melitensis (reference strain 16 M) and Brucella ovis as following (1020, 604), (980, 690), (950, 660), (414,870), (446,414) and (356,576) for the mentioned bacterial strains and enzymes (SDH-ase and ATP-ase in each bracket) respectively, in most studied species and strains of bacteria there were reverse relationships between SDH-ase and ATP-ase actual reactions. (the difference between O.D. of zero time and final reaction) the qualitative cytochemical study showed also differences between the different strains in order to strengthen our preliminary trial, a further complementary studies should be carried out in order to establishing a scientific table containing all the optical densities for the all bacterial enzymes, using the quantitative cytochemical studies for the all pathogenic bacteria (isolated and reference strains) as a new sole or confirmatory method for identification of future unknown samples of bacterial species or strains.

INTRODUCTION

Brucella melitensis cause, a highly contageous zoonotic disease caused by gram negative facultative intracellalor coccobacilli. Brucellosis induce acute disease (abortion storms, orchitis, mastitis, arthritis, synovitis in animals, and nervous signs in sheep and goats), the disease become then chronic and may be persist for life **(Wilkinson,1993)**. Ovine Brucellosis in sheep caused by Brucella ovis which cause infertility in rams (by chronic orchitis) and rarely infect ewes and rarely cause abortion, it may induce weak birth, stillborn lamb, placentitis **(Queensland**

Government, primary industries and Fisheries, 2011).

For identification of Brucella organisms, a presumptive evidence of Brucellae is provided by the demonstration by modified Acid-fast staining of organisms in aborted materials or vaginal discharges with serological methods, the polymerase chain reaction (PCR) provide additional means for detection. Brucellae should be isolated using plain or selective media by culture from uterine discharges, aborted foeti, udder secretions or from lymph nodes and reproductive organs of both sexes. Brucella species and biovars should be identified by phage lysis and by cultural, biochemical and serological criteria, PCR can provide both complementary and biotyping method based on specific genomic sequences **(OIE Manual, 2008)**. Concerning the serological tests for Brucella identification methods, Rose Bengal test and Buffered plate agglutination test, the complement fixation test, the enzyme linked immunosorbent assay (ELISA), or the Fluorescence polarization assay are suitable tests for screening herds and individual animals. However, no single serological test is appropriate in each and all epidemiological situations, so that the screening tests should be confirmed by complement fixation test and PCR techniques. In dairy herds, the direct ELISA or milk ring test used for bulk milk samples. Brucellin skin test may be used as screening or confirmatory herd test especially in unvaccinated herds. Interferon gamma tests, precipitation test that used native hapten antigen may showing promise in differentiating Brucellosis from exposure to other cross reacting microorganisms **(Bricker, B. J., 2002)**. Genetic and immunological evidence indicates that all members of the Brucella genus are closely related. The classical names related to the six Brucella nomen species are validly published in approved lists of bacterial names and the designed types strains are attached to these validly published names: Brucella abortus, B. melitensis, B.suis, B.neotomae, B.ovis and B. canis. Strains of Brucella have isolated in the last decade from marine mammals that cannot be described to any of the above recognized species **(Ewalt et al., 1994 and Foster et al., 2002)**.

Investigation are continuing to establish their correct position in the taxonomy of the genus and it is proposed that they could be classified into two new species: B. ceti and B. pinnipedialis **(Jensen et al., 1999; Cloeckaert et al., 2001 and Foster et al., 2002)**.

Plesiomonas shigelloides cause gastroenteritis but with a mild self- limiting disease with fever, chills, abdominal pain, nausea. In severe cases watery diarrhea become greenish yellow, foamy, non-mucoid and bloody tinged **(Bad Bug Book, BBB, 2009)**.

Salmonella dublin cause a typical disease in cattle, characterized clinically by septicemia, dysentery, inappetance, great depression pyrexia, depressed milk yield, mucoid fluidy diarrohaea which usually, contain blood clots and shreds of necrotic intestine and abortion. In calves, morbidity and mortality may reach 100%. Calves may found dead without any clinical signs, in some cases carpal and tarsal joints may be enlarged **(Richardson, 1974)**.

E. coli is normally lives in intestine of

human and animals. most types are harmless, but some E.coli can cause disease, enterotogenic E. coli is a group of E. coli produce a specific toxins that could stimulate intestine to cause profuse watery diarrhoea with abdominal cramping, fever, nausea, vomiting, chills, loss of appetite, headach, muscle arching and bloating **(National center for Immunization and respiratory disease - Bacterial diseases (2005)**.

The cytoplasmic membrane was first established as the site of respiratory enzymes and cytochrome linked electron transport of Bacillus megaterium **(Strock and Wachsman, 1957)**. The bacterial lipoprotein granuoles which reduce tetrazolium salt in the whole bacterial cells have been regarded as equivalent to animal and plant mitochondria and thus may be considered as bacterial mitochondria **(Mudd et al., 1961)** and the cytoplasmic membrane may equivalent to the Christae Mitochondriae of animal and plant cells **(Green, 1961)**.

The activity of succinic dehydrogenase (SDH-ase) enzyme was studied in E.Coli **(Sedar and Burde, 1965-a)** and in Bacillus subtilis **(Sedar and Burde, 1965-b)** using combined cytochemical and ultrastructural studies by electron microscopy, each organism was incubated in a substrate medium containing the tetranitroblue tetrazolium (TNBT) which served as an electron acceptor to be reduced into a violet bluish formazan pigment at the site of enzyme reaction that associated with the bacterial plasma membrane .

The adenosine - tri - phosphatase (ATP-ase) enzyme activity was located mainly in the cytoplasmic membrane, mesosomes and in the inner layer of cell wall facing the cytoplasmic membranes of Bacillus subtilis -168. The cytochemical reaction of ATP-ase was observed as fine dense granules incorporated into the membranes as high contrast lead precipitate on the membrane surfaces **(Chrerepova et al., 1986)**. A variable localization of ATP-ases in the different microbial species was established by **Voelz (1964)**. In E.coli the ATP-ase enzyme was found mainly in the cytoplasmic membrane and the cell wall, in Bacillus cereus, it was observed in the cytoplasmic membrane, cytoplasm and in the nuclear region of the cell, while in Myxococcus xanthus it was seen only in the cytoplasm. **Mikhaleva et al., (1984)** demonstrated the ATP-ase enzyme in the isolated membrane vesicles from E.coli by using a combination of cytochemical and electron microscopy, also **Kubak and Jotis (1981)** used negative staining and electron microscopy to prove the presence of ATP- ase in isolated membranes of Staphylococcus aureus, but **Kushnnarea et al., (1968)** studied the distribution of the ATP-ase enzyme in the intact cells of Staph. aureus and showed its localization in the cytoplasmic membranes, the mesosomes and the cross wall of dividing cells. In Bacillus subtilis the ATP-ase has been isolated, purified and characterized from the cytoplasmic membrane **(Ziegor and Monteil, 1982)**.

Mg-ATPase activity was determined in E.coli bacterial suspension after 10 minutes incubation with the reaction medium, 2.5N perchleric acid used for terminating the reaction and the amount of phosphate released was determined colormetri-

cally **(Butling et al., 1973 and Finke and Subbaraw, 1975)**.

The periodic acid thiocarbohydrazide silver proteinate reaction (Thiery methods) separates the bacteria into four large groups according to the production of silver grains at the site of the cell wall or cytoplasmic membrane or both in Corynbacterium, Mycobacterium and Nocardia. In Bacillus subtilis and Brucella organisms, only the cell wall reacts. In lactobacillus and microbacterium both the cell wall or cytoplasmic membrane reacts. In E.coli and Clostridium neither the cell wall nor the cytoplasmic membrane reacts.

Micro-organisms have been classified and identified on the basis of characteristics including morphological or staining **(Baely and Scott, 1978)**, Biochemical **(Cowan and Steel,1974)**, serological **(Oda et al., 1979)**. Recently there has been a tendency to determine the molecular genetic characteristics as polymerase chain reaction (PCR) analysis **(Sambrook et al., 1989)**, but such molecular genetic techniques are still time consuming, so that for routine identification of isolates, the ease of testing and the total completion time are critical.

Previous trial for quantitative cytochemical determination of Succinic dehydragenase reaction through estimation of the optical density of formazan pigment (the end product of the cytochemical enzyme reaction) in Bacillus megaterium by **(Hess and Dietrich, (1960)**. Moreover, this qualitative cytochemical staining method clearly separate the Mycobacterium leprae from certain leprosy -derived corynform bacteria **(Rastagi et al., 1984)**. the present study aimed for identification of the different species of bacteria through spectrophotometric (quantitative) and semi quantitative estimation of the optical densities of cytochemical reactions of SDH-ase and ATP-ase enzymes in six bacterial species and strains as a new preliminary trial for further identification or confirmation of the different species or strains of bacteria.

MATERIAL & METHODS

(A) Sampling (Koneman et al., 1992):

1- samples from milk, aborted foeti, mammary glands, lymph nodes of aborted sheep, goat and cattle and infected ram testicles were used for isolation, identification and typing of Brucella strains.
2- Samples from: milk of mastitic buffaloes and cattle, intestine of enteric calves, infected lungs for isolation and identification of E.coli.
3- Samples from infected goats, cattle, fish and contaminated water were used for isolation and identification of plesiomonas shigeloides.
4- Samples from the deep sepsis from cattle were used for isolation and identification of Salmonella dublin.
5- B. melitensis refcreence strain (16M).

(B) Isolation and identification of Bacteria:

1- The Brucella melitensis (Biovar3 field strain) and Brucella ovis were identified and biotyped according to **Alton et al., (1988)**.
2- The E.coli, Salmonella dublin and plesiomonas shigelloides were identified and biotyped according to the methods described by **Koneman et al., (1992)**.

(3) Preparation of bacterial suspensions:

The colonies from previously isolated and identified six different species or strains of bacteria (E.coli, Salmonella dublin, Plesiomonas shigelloides, Brucella melitensis (field isolated strain), Brucella melitensis (reference strain), and Brucella ovis were used for preparation of bacterial suspensions with a constant concentration of 15 X 10^8 CFU which prepared by Turbidity standards for each species or strain of studied bacteria according to **Quinn et. al. (1994)**.

(4) Preparation of substrate incubation medium for mitochondrial Succinic Dehydragenase (SDH-ase) enzyme: The succinate substrate incubation medium for the mitochondrial Succinic dehydrogenase was prepared according to the method recorded by **Nachlas et al., (1957)**.

(5) Preparation of substrate incubation medium for Mitochondrial Adenosine Triphosphatase (ATP-ase) (Mg^{2+} - activated, lead method):

The ATP- substrate incubation medium for mitochondrial ATP-ase was prepared according to the method described by **(Wachstein and Meisel, 1957)**, this method need normal saline solution for dilution and ammonium sulphide 1% to give the final colored product in solution after incubation.

Both bacterial suspension (15X10^8 CFU) and the substrate incubation media for the two enzymes are freshly prepared.

(6) Recording the Optical Densities (O.D.) For the Bacterial Suspensions:

For each species or strain of bacteria put 1 ml of bacterial suspension (15x10^8 concentration) in each of five, dry sterile, clean test tube and record their O.D. using spectrophotometer at wave length (WL) of 555nm for all bacterial suspension of the studied species and strains according to **Anon and Stieckenius (1974)**, take the mean ± SE, then repeat such method for the all studied species and strains of bacteria.

(7) Procedure for Quantitative Determination of Bacterial Succinic Dehydrogenase (SDH-ase) Enzyme activity adopted from (Nachlas et al., 1957):

1- In five clean, sterilized and dry test tubes put 1 ml of bacterial suspension of each bacterial species and strain, then add 1 ml of succinate substrat incubation medium for SDH-ase enzyme reaction in each tube, directly after mixing record the O.D. of the mixture (at WL= 450nm) this step (zero starting reaction time) is very important for recognizing the occurrence of the actual SDH-ase reaction after incubation.
2- Incubate the above mixtures (in test tubes) in the incubator for 1 hour at 37°C and take the mean O.D.± SE
3- After incubation period record the O.D. (at WL= 450 nm) of bacterial SDH-ase enzyme.
4- Repeat the above mentioned methods for each species and strains of bacteria.

(8) Procedure for Quantitative Determination of Bacterial ATP-ase Enzyme Reaction (Mg^{2+}-activated, Lead Method) for different species (strains) of Bacteria (Adopted from Wachstein and Meisel, 1957):

1- In five clean, dry and sterile test tubes,

put 1ml of bacterial suspension of certain species of bacteria in each tube.

2- Add 1 ml of substrate incubation medium for mitochondrial ATP-ase enzyme in each tube with gentle mixing then directly record the optical density of such mixture (before enzyme reaction= zero or staring reaction time)

3- Incubate the above mixture (bacterial suspension + ATP-incubation medium) in the incubator at 37°C for 1 hour.

4- After incubation period add 0.5 ml of ammonium sulphide 1% solution in each tube with gentle mixing (the colour of the mixture become dark brown).

5- Add 4.5 ml of the normal saline solution in each tube with gentle mixing for diluting the mixture.

6- Record the optical density (O.D.) of diluted mixture for each tube (that containing the brown end product of the final ATP-ase enzyme reaction, the lead-sulphide) at 555 nm wave length according to **Andreu et al.,(1973)**.

7- Repeat the above mentioned methods for the other species and strains of bacteria and recording the O.D.as (mean ± SE) of ATP- ase enzyme reactions.

(9) The Semiquantitative Histochemical Techniques:

The same histochemical techniques for SDH- ase and ATP- ase enzyme applied for frozen sections of animal tissues according to **Nachlas et al., (1957) and Wachstein and Meisel (1957)** respectively which could adapted for bacterial suspensions to be applied with ($15x10^8$ CFU) of bacterial cells concentration, where equal volumes of 0.7ml of bacterial suspensions (of the each of the six species and strains of bacteria) and the specific incubation medium for each enzyme on clean, dry and sterilized glass slides to be incubated at 37c° for 1 hr. In case of SDH-ase enzyme reaction the slides left to drying after incubation then counter stained with 2% ethyl green solution then dried and mounted with few drops of glycerol jelly and covered with cover slide for microscopical examination. In case of ATP-ase enzyme reaction additional step (after incubation and drying) is adding of 1% ammonium sulfide, left to drying, counter stained with 0.5% safranin-o, dry and mounting with glycerol Jelly, and covered with cover slids to microscopical examination. the semi-quantitative determination of the amount of bluish violet formazan granuoles or pigment(in case of SDH-ase reaction) and semiquantitative estimation of the amount of the lead sulfide granuoles or pigment in case of ATP-ase reactions. The two enzymes are semi-quantitatively described from traces (±) to intense (+++++) reaction accordding to **Pearse (1972)**.

(10) Statistical Analysis:

The obtained data were statistically evaluated using F-test and T-student test according to **Snedecor and Cochran, (1969)**.

RESULTS & DISCUSSION

(1) Bacterial isolations and identifications:

Five isolates could be identified as: Brucella melitensis (Biovar3 field strain), Brucella ovis, E.coli, Plesiomonas shigelliodes and Salmonella dublin. The biotypes of the isolated bacteria and the obtained reference strain of Brucella melitensis are tabulated in table (1):

(2) The Optical Densities (O.D.) of Bacterial Suspensions (15×10^8) of Different Species And Strains of Bacteria:

The least O.D. of bacterial suspensions was showed by Brucella ovis (255), Brucella melitensis (reference strain) (272) then Salmonella dublin (38.8). The medium O.D. were showed by Plesiomonas shigelloides (654), Brucella melitensis (Biovar3 field strain) (676). The highest O.D. was showed by E.Coli (706). There were significant difference of O.D between the all species and strains of bacteria ($P < 0.05$) (Table 2).

(3) The O.D. of Bacterial Suspension (15×10^8) at Starting Zero Reaction Time Before Adding Succinate substrate for Succinate dehydrogenase Enzyme Reaction:

All O.D. of bacterial suspensions were recorded at the Zero time before incubation essentially to know the occurrence of the biochemical reactions of enzymes for each of the diluted 6 bacterial suspensions as follow: 197, 324, 335, 348, 245 and 212 for: Salmonella dublin, Plesiomonas shigelloides, E. Coli, (field isolated strain), Brucella melitensis (Biovar3 field strain), Brucella melitensis (reference strain) and Brucella ovis respectively and showed lower O.D than that of the initial bacterial suspensions.(table 3).

(4) The O.D. of Bacterial Suspensions After SDH-ase Enzyme Reaction :

After incubation period of bacterial suspensions with the substrate medium for SDH-ase enzyme. All the bacterial suspensions of the different species of bacteria showed significant increase of optical densities of SDH-ase activities than that before incubation (starting zero reaction time) but with different levels of enzyme activities according to the bacterial species or strains, there were significant differences between the all six studied bacterial species and strians. The highest O.D. of SDH-ase activity were showed by E.Coli (1020) followeed by Brucella meletensis (980) then Plesiomonas shigelloides (950), the lower O.D. of SDH-ase activity was showed by Brucella ovis (356), Salmonella dublin (414), then Brucella melitensis reference strain (446). (Table-3).

When comparing between the differences between the O.D. before and after (B-A) SDH-ase incubation, we could arrange the bacterial species and strains from the higher to the lower SDH-ase activities (the actual enzyme reactions) as following: (1) E. coli (the higher SDH-ase activity) (2) Brucella melitensis (isolated), (3) Plesiomonas shigelloides, (4) Salmonella dublin, (5) Brucella melitensis (reference strain) then(6) Brucella ovis (the lower SDH-ase activity), as shown in (Table 5).

(5) ATP-Ase Activity Before Incubation (At Starting Zero Reaction Time):

There were significant differences between the means of the optical densities of ATP - ase of the different studied bacterial speciees and strains before incubation at Zero (starting) reaction time (Table. 4).

(6) The O.D. of Bacterial Suspension After Incubation With ATP - Substrate Medium:

There were very highly significant ($P \le 0.001$) of the O.D of ATP ase activities of different species and strains of bacteria after incubation period than that before incubation,

but there were significant difference ($P \leq 0.05$) between the means of the O.D of ATP-ase activities between the all six studied species and strains of bacteria, the higher O.D. of ATP-ase activities were showed by Salmonella dublin, Brucella melitensis (isolated field strain) then E. Coli, but the lower O.D. of ATP-ase activities were showed by Brucella melitensis (reference strain) and Brucella ovis (Table-4) .

When comparing the differences between O.D. of ATP-ase activities after and before (A-B) incubation period (the actual enzyme activity) we could arrange the bacterial species and strains according to the higher ATP- ase activities to the lower ones as following (1) Salmonalla dublin (The higher ATP - ase activity (2) Brucella ovis, (3) Brucella melitensis (reference strain) (4) Plesiomonas shigelloides, (5) Brucella melitensis (Biovar3 field strain) then (6) E.coli (the lower ATP - ase activity) (Table -5).

(7) Relationships between the Actual activities of bacterial SDH - ase and ATP - ase :

Approximately there were indirect relationships between the activities of SDH - ase and ATP-ase of the studied bacterial species and strains according to the percentages of the differences between the O.D. after and before (A-B) incubation periods with their specific substrate media (except that of Brucella melitensis, reference strain, where the two enzymes with the same percentage), such indirect relationship pointed to that when activity of SDH-ase was increased, the activity of ATP-ase was decreased, in the same species or strain of bacterium as shown in (Table-6) and Fig (1) .

(8) Semiquantitative cytochemical Determination of SDH-ase and ATP-ase Enzyme Reactions:

(a) SDH-ase Enzyme Activity:

SDH-ase reactions showed: Intense, moderate to strong, moderate to strong, submoderate, submoderate and weak to submoderate reactions in : E. coli, Brucella melitensis (Biovar3 field strain), Plesiomonas shigelloides, Brucella melitensis (Ref. strain), Salmonella dublin and Brucella ovis respectively.

(b) ATP- ase enzyme Reaction:

The ATP-ase enzyme reactions showed: moderate, strong, moderate to strong, weak to submoderate, intense and submoderate to moderate enzyme reaction in: E. Coli, Brucella melitensis (Biovar3 field strain), Plesiomonas shigelloides, Brucella melitensis (Ref. strain), Salmonella dublin and Brucella ovis respectively, as shown in (Table-7) and fig.(2).

We try through the current study to establishing a new growing approach for identification of bacterial species and strains, such method depends on the quantitative estimation of bacterial enzymes spectro photometrically after incubation with their specific substrates through the present cytochemical techniques that adapted for bacterial suspensions, such method also carried out under fixed concentration and other factors for all studied species and strains of bacteria **(Wachstein and Meisel, 1957 and Nachlas et al., 1957)**. Previous cytochemical techniques for bacterial Succinic dehydrogenase (SDH-ase) enzyme localization in E. Coli and Bacillus subtilis **(Sedar and Burde, 1965, a & b)** and adenosine triphosphatase (ATP - ase) enzyme localization in Bacillus subtilis -

168 **(Chrerepova, 1986)**, and in E. Coli **(Vociz, 1964) and Mikhaleva et al., (1984)** but without quantitative estimation of the enzyme reactions, only the workers aimed to localization of the enzymes using combined cytochemical and electron microscopy studies, for establishing a basic knowledge on bacterial enzymes.

The semiquatitative histochemical method was previously used for identification of bacterial species, the cytochemical detection of oxidase activity in the bacterial cells might assist in the rapid presumptive identification of some bacterial species isolated from wounds, cerebrospinal fluids and blood cultures after adding dimethyl p-phenyl enediamine Hcl and α-naphthal to a hanging drop of bacterial suspension. Saw blue granuoles in the rods of bacillus anthracis as acytochemical methods for identification of such species **(Cooper, 1969)**.

Pulse-field gel- electrophoresis has been developed that allows the differentiation of several Brucella species **(Charach et al., 1997 and Jensen et al., 1999)**. Brucella biotyping and distinguishing vaccine strains by PCR can be accomplished satisfactory but there has been limited validation of PCR for primary diagnosis. The first species- specific multiplex PCR-assay for differentiation of Brucella was described by **Bricker and Halling, 1995**. The assay named AMOS-PCR was based on the polymorphism arising from species -specific localization of the insertion sequence IS711 in the Brucella chromosome and comprised five oligonuclotide primers that can identify and differentiate B. abortus biovars 3,5,6 and 9. the modification to the assay designated Bass-PCR have been introduced over time to improve performance and additional strain-specific primers were incorporated for identification of Brucella **(Bricker and Halling, 1985 and Ewalt and Bricker, 2000)**.

Considering the serological identification of Brucella in all epidemiological situations, it should be stressed that the serum agglutination test (SAT) is generally regarded as being unsatisfactory for the purposes of international trade, the complement fixation test (CFT) is diagnostically more specific and standardized than SAT, some enzyme linked immunosrbent assays (ELISAs) and Fluorescence polarization assay (FPA) are comparable with or better than the CFT in addition to its simpler to perform and more robust **(Nielsen et al.,1964)**.

Previous trial for quantitative estimation of SDH - ase enzyme activity by colormetric determination of the optical density after incubation with the succinate substrate medium with Bacillus megaterium bacterial suspension of certain concentration by **Hess and Dietrich (1960)** without using such method for SDH-ase reaction by other workers as a method for bacterial identification according to our available data till February (2011).

Our trial was carried out for obtaining the optical densities of six species and strains of bacteria for the two studied enzyme (ATP- ase and SDH - ase), which are members of oxidative phosphorylation enzyme reactions and energy metabolism in bacteria **(Cavari et al., 1968)**. The difinit mean values of the different spectrophotometrically determined bacterial

enzymes should be diagnostic for bacterial species under fixed factors of the cytochemical and bacteriological techniques for the all the studied bacterial species and strains, so that similar future complementary study should be carried out to establishing a scientific table recording the optical densities of all bacterial enzyme activities for the all species and strains of pathogenic bacteria (isolated and reference strain) under the same standardized cytochemical and bacteriological methods for future identification of unknown species and strains of bacteria.

There are suitable screening tests as : Rose Bengal test (RBT) and Buffered acidified plate antigen test (BAPAT) as well as ELISA and FPA, the positive reactions should be retested using a suitable confirmatory strategy **(Wright et al., 1997)**.

The current study started to recording the optical densities of bacterial suspensions of the different studied species and strains of the same bacterial concentration (15×10^8), a similar study for recording optical density of bacterial suspension of Bacillus megaterium by **Hess and Dietrich (1960)** and such optical densities of the bacterial suspensions of all pathogenic bacteria and their strains should be recorded also in the supposed scientific table of enzyme activities for strengthen or supporting the bacterial identification. The bacterial density in the bacterial suspension was previously recorded also by means of a klett photocolorimeter using a green filter **(Herrera and Garcia, 1972)**.

We recorded also the optical densities of bacterial suspensions directly after adding equal quantities of substrate media for each of the two studied enzymes, this step is very important for recording the starting time (zero time) of the enzyme reaction before the period of incubation in order to recognize the occurrence of the enzymatic reaction, as if there was no enzyme reaction occur (for any wrong in preparation of substrate medium or in the cytochemical technique) the optical density remain approximately the same after incubation period, in contrast the significant increase of the optical density after the period of incubation than that before incubation and this change indicated the successful of the enzyme reaction which then recorded after exactly 1 hour incubation for all studied bacterial species and strains, and the difference between optical density after and before incubation period should be considered the actual enzyme activity in each type of bacteria. The results of the present study indicated presence of significant increase of the optical densities of both SDH-ase and ATP-ase enzyme reactions after incubation period than that before incubation in all studied species and strains of bacteria.

The current work revealed that there were significant differences of optical densities of the two studied enzymes after enzymatic reactions with their substrates between the six studied species and strains of bacteria, this indicated that there were significant variations in the levels of enzyme activity between the studied species and strains of bacteria.

The present results recorded that the higher SDH - ase enzyme reactions were showed by E. Coli and plesiomonas shigelloides, and the lower SDH - ase enzyme activities were

showed by Brucella ovis and Salmonella dublin. The higher or lower SDH - ase enzyme activity may be correlated with the activities or pathogenicity of such bacteria considering the role of such enzymes in bacterial biochemistry or physiology. The SDH - ase is the only membrane - bound enzyme of tricarboxylic acid (TCA) cycle in E. Coli converting succinate to fumarate **(Marr, 1960)** and therefore, the enzyme participate in respiration (oxidation). **Herrera (1968)** suggested that the carbon-growth substrate was important in governing the synthesis of SDH - ase in E. Coli. The SDH-ase enzyme appears to play an important respiratory (oxidative) role since amytal (NADH - oxidase inhibitor) inhibited growth only slightly when the succiante was used as carbon source as compared to the strong inhibition of the growth when glucose was used as carbon source **(Herrera and Garcia, 1972)**, but **Cavari et al., (1968)** found that the activity of SDH - ase enzyme increased about sixfold when E. Coli was grown in a succinate - containing medium as compared to the activity of the enzyme shown by bacteria grown in a medium containing fumarate or mannitol.

The present results revealed that the adenosine - tri phosphatase (ATP - ase) enzyme was significantly increased in all studied species and strains of bacteria after incubation period than that before incubation, also it was found that the higher ATP-ase reactions were recorded in Salmonella dubin, but the lower ATP-ase reaction were detected in Brucella melitensis (reference strain) and Brucella ovis. According to our knowledge, no available data could be detected about the quantitative analysis of mitochandria ATP-ase activity in bacteria, so that further study should be carried out on the relation of enzyme activity (higher or lower) with the activities, pathogenicity or virulence of bacteria considering the role of mitochondrial ATP - ase in breakdown of the ATP molecules and energy metabolism in bacteria.

The present qualitative cytochemical study revealed that the end product of mitochondrial ATP-ase enzymatic reaction as dense granules of lead sulfide as shown in figures (1:C & D), similar cytochemical studies could be obtained by **Goldfischer et al., (1964) and Cherepova et al., (1986)**.

From the obtained data, we should consider that the actual enzyme activity was the difference between the optical density after incubation and that before incubation period of the enzyme with its specific substrate medium, so we could arrange the studied bacterial species and strains according to the actual SDH-ase enzyme reactions from the higher to the lower reaction as: (1) E.Coli (the higher reaction) (2) Brucella melitensis (Field st.) (3) Plesiomonas shigelloides, (4) Salmonella dublin (5) Brucella melitensis (reference strain) and (6) Brucella ovis (the lower SDH-ase reaction). But in case of actual ATP-ase reaction we could approximately obtained the reverse arrangement to that of actual SDH - ase reaction as (1) Salmonella dublin (the higher ATP-ase activity) (2) Brucella ovis, (3) Brucella melitensis (reference strain) (4) Plesiomonas shigelloides (5) Brucella melitensis (Field st.) and (6) E.coli (the lower ATP-ase reaction). When we relate each of the actual enzyme activity of any species or strain of bacteria to the higher actual enzyme activity

for obtaining percentages and arrange such percentages from higher (100%) to lower, so a clear reversible relationships between SDH-ase and ATP-ase enzyme reaction of the six studied bacterial species and strains of bacteria could be obtained as illustrated in figure (2).

It should be differentiate between two types of bacterial ATP-ase enzymes, the first is the membranous ATP-ase with the role of ion transport and membrane permeability **(Wittman, 1961)** and the second is the mitochondrial ATP-ase with oxidative phosphorylation function **(Goldfishea et al., 1964)**. The reverse relationships between the activities of SDH-ase and ATP-ase indicated that such reactions were carried out in bacterial mitochondria, where the SDH-ase reaction is activated by presence of both succinate and ATP-molecules, as the lack of ATP (due to ATP-ase enzyme activation) and the excess of oxaloacetate leading to suppression of SDH-ase enzyme reaction in bacterial and plant cells as recorded by **(Hung et al., 2010)** and others.

There were many methods for identification of bacterial species and strains such as the biochemical methods **(Cowan and Steel, 1974)**, serological methods **(Oda et al., 1979)**, morphological characters (staining) **(Baely and Scott, 1978)**, polymerase chain reaction (PCR) for bacterial nucleic acids **(Sambrook et al., 1989)**, and other methods.

Brucella organisms could isolated in tryptose soya agar, and Brucella gar, the serological detection of antibodies by rose bengal test, ElISA, 2-mercaptoethanol, tube agglutination test, and confirmed by complement fixation test.

Considering the identification of Brucella organism, any colonies of Brucella morphology should also be checked using gram- or stamp-stained smears. As the serological properties, dyes and phage sensitivity are usually altered in the non-smooth phages, attention to the colonial morphology is essential in typing tests, the biochemical identification of Brucella organisms by urease, oxidase and catalase, also the serological identification by slide agglutination test with anti- Brucella polyclonal serum. The species and Biovar identification with A-, M- or R. specific expertise in these methods, the use of several phages (e.g. Tbilissi (Tb), Weybridge (Wb), Izatnagar (IZ) and(R/C) provides a phagotyping system for practical identification of smooth and rough species of Brucella **(Alton et al., 1988)**.

The recently development of PCR provides an additional means of detection and identification of Brucella sp. **(Bricker and Halling, 1995)** despite the high degree of DNA-homology within the genus Brucella, several molecular methods including : PCR, PCR restriction fragment length polymorphism (RFLP) and southern blot have been developed which allowed to a certain extent to differentiation between Brucella species and some of their biovars **(Bricker, 2002 and Moren et al., 2002)**.

Plesiomonas shigelloides is a gram negative rods - shaped bacteria isolated from fresh water and its fish, shellfish, cattle, goats, pigs, cats, dogs and monkeys, it is placed among

enterobacteriaceae and antigenically similar to shigella which cross reacted with Shigella sonnei, but it can differentiate from Shigella in diarrhoeal stool and Shigella is oxidase positive, also plesiomonas is DNA-ase negative. Plesiomonas also should be distinguished from Aeromonas by biochemical tests **(Niedziela et al., 2002)**.

Salmonella dublin isolated from feces, milk and from aborted foeti **(NADIS, 2007)**. Taxonomy of Salmonella dublin as gamma proteobacteria, enterobacteriaceae (Salmonella enterica), subspecies (enterica), serover (dublin) Salmonella dublin (strain CT - 020 21853) is a bovine adapted serover that is genetically related to Salmonella enteritidis that live in bovine intestine **(Ravel et al., 2008)**.

The enzyme β-glucuronidase using the substrate methyl umbeliferyl glucuronide (MUG) in the agar or broth media is a test for E. Coli 0157 strains, the reaction give a fluorescent product which could be detected by long wave UV-light, so the E.coli 0157: H7 and the non-motile E. coli 0157 strains that produce shiga-like toxin was lack such enzyme that are MUG-negative, such MUG-assay may be used in conjunction with testing for sorbitol fermentation and agglutination in E. coli 0157 - antiserum is considered a useful screening test for toxigenic strains of E. coli 0157 **(Thompson et al., 1990)**.

Based on the current study, it could be obtained six significantly different optical densities for each of the two mitochondrial enzymes activities for the six studied species and strains of bacteria as following: The optical densities of both (succinic dehydrogenase & ATP-ase enzymes) for E. coli, Brucella melitensis (Field st.), plesiomonas shigelloides, Salmonella dublin, Brucella melitensis (reference strain) and Brucella ovis are (1020, 604), (980, 690), (950, 660), (414, 870) (464, 414) and (356, 576) respectively, for (SDH-ase & ATP-ase respectively in each bracket), and there were reverse relationships between the actual reactions of the SDH - ase and the ATP - ase enzymes in most bacteria. Further complementary studies for including all pathogenic bacteria (isolated and reference strains) and all bacterial enzymes for further quantitative cytochemical determination of bacterial enzymes using standardized cytochemical and bacteriological methods to preparing complete scientific table for future identification of unknown samples of bacterial species or strains as a new sole or as a further confirmatory methods for bacterial identification.

Table (1): The different Bacterial isolated and reference strains with their biotypes (family or colony shape):

Bacterial isolates	E.Coli	Salmonella Dublin	Plesiomonas Shigelliodes	Brucella melitensis (field St.)	Brucella ovis	Brucella melitensis (references Strain 16M)
Serotypes	0157:H7	Biotype-D	E.Plesiomonas	Biovar-3 (Smooth field strain)	Rough type	Biovar-1, (smooth type)

Table (2) : The Optical Densities (O.D) of The Bacterial Suspensions (15 X 10^8) of The Different Species and strains of Bacteria (At WL = 555nm).

Species of Bacteria	Salmonella dublin	Plesi-omonas Shigell-oides	E.Coli	Brucella meliten-sis Field st.	Brucella melitensis (Ref.St.)	Brucella ovis	LSD P (≤ 0.05)
O.D of	A 386	B 654	C 706	D 676	E 275	F 255	5.152
Bacterial	±	±	±	±	±	±	
Suspension	4.12	3.30	3.100	3.100	4.80	3.42	

N.B: (1) LSD= Least Significant Differences between means (at P ≤ 0.05) (2) The different litters in the row denote presence of significant difference between the O.D. of the different species (at p ≤ 0.05).

Table (3) : The quantitative estimation of the succinic dehydrogenase (SDH – ase) enzyme activities in the different species of bacteria (At wL = 450 nm).

Species of Bacteria	Salmonella dublin	Plesiomonas Shigelloides	E.Coli	Brucella melitensis Field st.	Brucella melitensis (Ref.St.)	Brucella ovis	LSD P (≤0.05)
O.D before incubation (zero time)	A 197 ± 2.95	B 324 ± 3.30	C 335 ± 2.67	D 348 ± 2.42	E 245 ± 1.78	F 212 ± 2.42	5.875
O.D of SDH-ase enzyme activity after incubation	A 414 ± 2.04 ***	B 950 ± 3.16 ***	C 1020 ± 2.28 ***	D 980 ± 1.65 ***	E 446 ± 1.65 ***	F 356 ± 2.28 ***	2.694

N.B: (1) LSD = least significant difference between means(at P ≤ 0.05). (2) -The different litters in rows denote presence of significant difference between the means (at P ≤ 0.05) (3) *** = presence of very-highly significant difference between each of two means before and after incubation in each type of bacteria (at P ≤ 0.001).

Table (4): The Quantitative Estimation of The Adenosine – Tri Phosphatase (ATP-ase) Enzyme Activities of The Different Species and strains of Bacteria (At WL= 565 Nm)

Species of Bacteria	Salmonella dublin	Plesiomonas shigelloides	E.Coli	Brucella melitensis (field St.)	Brucella melitensis (Ref.St.)	Brucella ovis	LSD P (≤0.05)
O.D of ATP – ase enzyme before incubation	A 295 ± 1.65	B 520 ± 2.28	C 574 ± 2.61	D 580 ± 2.21	E 246 ± 1.27	F 240 ± 3.16	5.143
O.D of ATP- ase enzyme activity after incubation	A 870 ± 2.04 ***	B 660 ± 1.90 ***	C 604 ± 2.04 ***	D 690 ± 1.79 ***	E 414 ± 2.43 ***	F 576 ± 3.06 ***	2.064

N.B: 1- LSD = least significant difference between means(at P ≤ 0.05). (2) -The different litters in rows denote presence of significant difference between the means (at P ≤ 0.05) (3) *** = presence of very-highly significant difference between each of two means before and after incubation in each type of bacteria (at P ≤ 0.001).

Table (5): Arrangement of SDH–ase and ATP-ase enzyme activities from higher to lower levels according to the differences between the means of the optical densities after and before (A-B) (The actual enzyme activity)

↓ Actual Activity	Number →	1	2	3	4	5	6
SDH–ase	Species	E.Coli	Brucella melitensis Field st.	Plesiom. shig.	Salmonella dublin	Brucla Melitensis (reference strain)	Brucella ovis
	(A-B) Incubation	685	632	626	217	201	144
ATP- ase	Species	Salmonella dublin	Brucella ovis	Brucella Meliteusis (ref. Str.)	Plasiom. Shig.	Brucella meliteusis	E.Coli
	(A-B) Incubation	575	336	168	140	110	30

N.B = (A – B) = O.D. after incubation – O.D. before incubation

Table (6): Relationship between the percentages of the actual bacterial enzyme activities between SDH–ase and ATP–ase enzymes in relations to their higher actual enzme activities .

→ Bacterial species / ↓ Enzymes	E.Coli	Brucella melitensis (Field St.)	Plesiom. shigel.	Salmonella dublin	Brucella melitensis (Ref.strain)	Brucella ovis
SDH – ase (%)	100	96.1	91.4	31.7	29.3	21
ATP-ase (%)	5.22	19.13	24.4	100	29.2	58.4

Table (7): The Semiquantitative Histochemical (Cytochemical) Estimation of Bacterial SDH-ase and ATP-ase Enzyme Reactions in Different Species and Strains of Bacteria.

Bacterial species → / ↓ Enzymes Activity	E.coli	Brucella melitensis Field st.	Plesiom. shiggel.	Salmonella dublin	Brucella melitensis (Ref.st.)	Brucella Ovis
SDH – ase	+++++	+++±	+++	++	++	+±
ATP-ase	+++	++++	+++±	+±	+++++	++±

N.B. ± = Trace reaction, + = weak reaction , ++ = submoderate reaction +++ = moderate reaction, ++++ = strong reaction, +++++ = Intense reaction

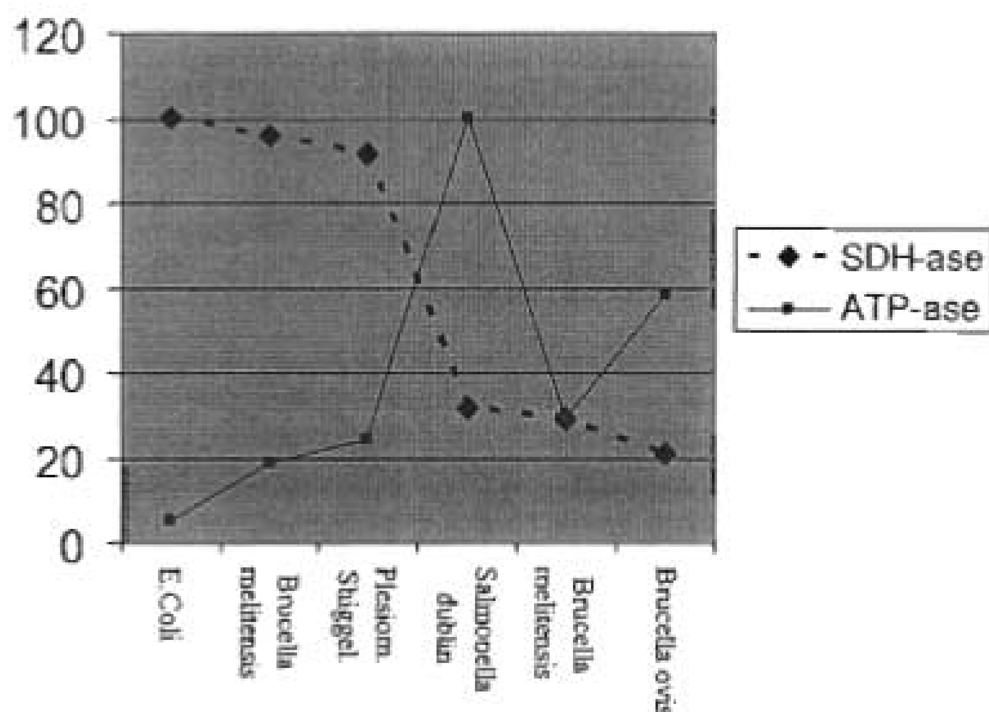
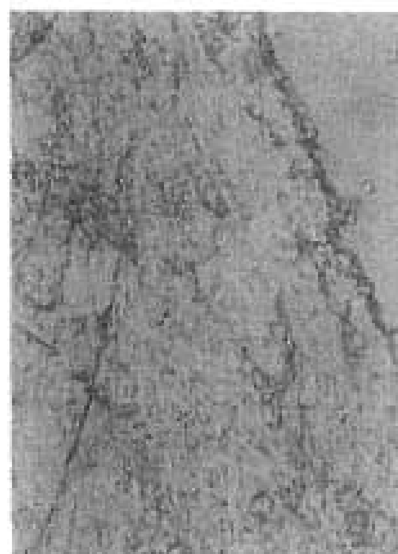
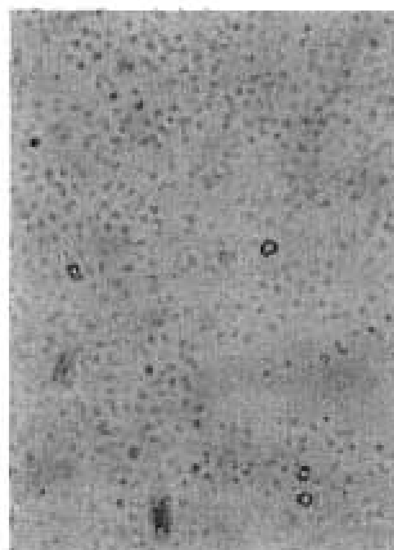


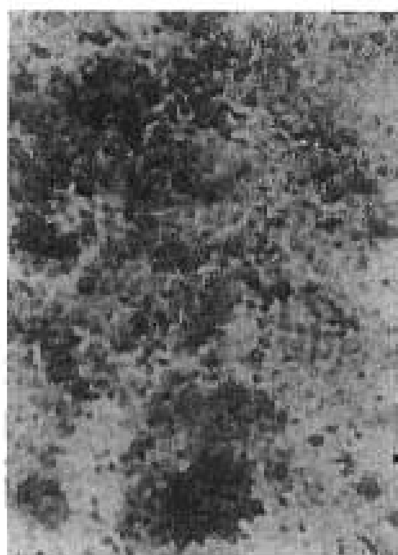
Fig. (1) : The reverse relationships between the percentages of the actual bacterial enzyme activities between SDH-ase and ATP-ase enzymes in relations to their higher actual enzyme activities.



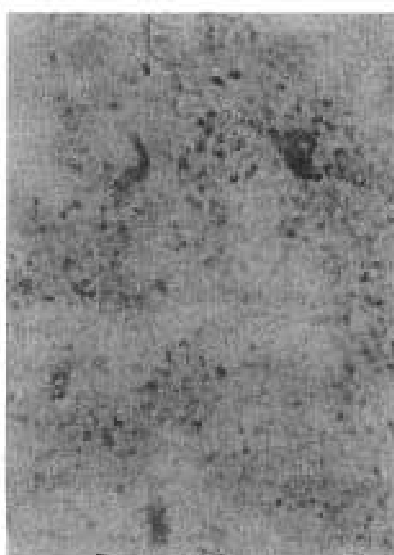
(1-A)



(1-B)



(1-C)



(1-D)

Fig (2): Showing The SDH-ase (1-A&1-B) and ATP-ase (1-C & 1-D) enzyme reactions in bacteria after incubation with their specific substrates as following: (1-A): intense (+++++) SDH-ase enzyme reaction in E.coli. (1-B): weak to submoderate (+ ±) SDH-ase activity in Brucella ovis. (1-C): intense (+++++) ATP-ase enzyme activity in Salmonella dublin. (1-D): weak to submoderate (+ ±) ATP-ase enzyme activity in Brucella melitensis (ref. St).(X 400)

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الملخص العربى

محاولة التعرف على العترات المختلفة من ميكروب البروسيللا مقارنة بأنواع البكتريا الأخرى باستخدام التقدير السيتوكيميائى الكمى لبعض انزيمات الميتوكوندريا

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يوجد عدة طرق للتعرف على أنواع وعترات البكتريا المختلفة كالطرق البيوكيميائية والسيرولوجية والأشكال الظاهرية (الصفات) وتفاعل البلمرة المتسلسل وطرق أخرى، ومن خلال هذه الدراسة تم عمل محاولة لتقييم طريقة التقدير الهستوكيميائى) الكمى والوصفى والحورة للاستخدام مع الخلايا البكتيرية من أجل قياس نشاط بعض انزيمات الميتوكوندريا للحصول على الكثافات الضوئية لتفاعلاتها (باستخدام جهاز الاسبكتروفوتوميتر) وذلك على ستة أنواع وعترات من البكتريا مع تثبيت كل ظروف التفاعلات لكل من إنزيى السكسينيك ديهيدروجينيز، والأدينوزين تراى فوسفاتيز (SDH-ase & ATP-ase).

ولقد أمكن الحصول على قراءات (مختلفة معنوياً) من الكثافات الضوئية (O.D.) لكل من (SDH-ase & ATP-ase) للستة أنواع من العترات البكتيرية تحت الدراسة وهى :

الميكروب القولونى الإى كولاى، البروسيلا ميليتنسيس (العترة المعزولة)، والبليشوموناس شيجيللويدس، والسلمونيللا دبلن، والبروسيللا ميليتنسيس (العترة المرجعية)، والبروسيللا أوفيس، وكانت الكثافات الضوئية للإنزيمين فيها كالآتى : (SDH-ase ١٠٢٠، ATP-ase ٦٠٤)، (٦٩٠، ٩٨٠)، (٩٥٠، ٦٦٠)، (٤١٤، ٨٧٠)، (٤٤٦، ٤١٤)، (٣٥٦، ٥٧٦) على التوالى، وكذلك وفى معظم الأنواع والعترات فإنه توجد علاقة عكسية بين النشاط الحقيقى لإنزيى (SDH-ase و ATP-ase) على التوالى بين الأقواس، وكذلك أوضحت نتائج الدراسة السيتوكيميائية النوعية وجود اختلافات ظاهرة بين تفاعلات الإنزيمين فى أنواع وعترات البكتريا المختلفة، ومن أجل تدعيم هذه الدراسة المبدئية فإنه يجب عمل دراسات مكملة لتكوين جدول علمى يحتوى على كل الكثافات الضوئية لكل الإنزيمات فى كل أنواع وعترات البكتريا المرضية باستخدام التقدير السيتوكيميائى الكمى كطريقة جديدة أو كطريقة تأكيدية للتعرف على أنواع وعترات البكتريا المختلفة المجهولة فى العينات.