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USING OF TRADITIONAL AND QUANTITATIVE CYTOCHEMICAL METHODS FOR IDENTIFICATION AND ENZYME CHARACTERIZATION OF SOME *E.COLI* SEROGROUPS CAUSING ENTERITIS IN BUFFALOES

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ABSTRACT

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Enteritis is most fatal disease especially for neonatal and young buffalo calves. Out of 150 fecal samples, 100 from diarrheic and 50 from non diarrheic (apparently healthy) buffalo calves were collected from private farms for isolation and identification of *E.coli* using morphological, cultural, biochemical and serological examination. Further cytochemical enzyme characterization of the different identified serogroups were carried out through the cytochemical quantitative determination of bacterial mitochondrial Adenosene-tri phosphatase (ATP-ase) and succinic dehydrogenase (SDH-ase) enzyme activities for their possible variations among the isolated and identified *E.coli* serogroups. Four values to each of *E.coli* serogroup (2 for each enzyme) as optical densities (OD.) could be obtained : (ATP-ase activity & Actual ATP-ase reaction and SDH-ase activity & Actual SDH-ase reaction) for *E.coli*-serogroups: *E.coli* O_{26}, *E.coli* O_{55}, *E.coli* O_{78}, *E.coli* O_{86} and *E.*coli O_{111} as following: (0.595 & 0.285 and 0.560 & 0.210), (0.565 & 0.265 and 0.550 & 0.200), (0.590 & 0.255 and 0.540 & 0.190), (0.590 & 0.205 and 0.490 & 0.180) and (0.600 & 0.160 and 0.560 & 0.150) nm respectively. It was observed that the SDH-ase enzyme activity differentiate between the majority of the studied *E.coli*-serogroups for the significant ($P \leq 0.05$) variations between their enzyme activities (except between the serogroups *E.coli* O_{26} and *E.coli* O_{111} which showed the same (non-significant) SDH-ase activity, but ATP-ase enzyme activity could not differentiate between the different *E.coli* serogroups because of its non-significant variations among different serogroups (except with the serogroup *E.coli* O_{55} which showed the significant lower ATP-ase enzyme activity and noted to be lower pathogenic than the other *E.coli* serogroups), so that the actual ATP-ase enzyme activities (the difference between the OD. after and before incubation period) may be used for differentiation between different serogroups. Further cytochemical characterization to other bacterial enzymes and to other *E.coli* serogroups should be carried out as additional tool for a definite differentiation and characterization of *E.coli* serogroups causing enteritis in buffalo calves.

***Keywords**: E.coli serogroups, diarrhea, cytochemical examination, bacterial ATP-ase and bacterial SDH-ase*

INTRODUCTION

Diarrhea is a predominant cause of mortality in neonatal buffalo calves leading to significant economic losses Rana *et al*. (2012). A key role in the etiology of bacterial infectious diarrhea in buffalo calves has been attributed to enteropathogenic *E.coli* mainly during the first three weeks of life Zaman *et al*. (2006). The pathogenic *E.coli* adhere to the mucosa and proliferate in the lumen of intestine, producing a potent enterotoxin, which stimulate excessive secretion of fluid from intestinal mucosa. This loss of fluid causes the principle sign (diarrhea) and often leads to dehydration and high rate of death in the buffalo calves and consequently causes heavy economic losses Radostits *et al*. (1994). *E.coli* produces septicemia and diarrhea in a wide range of hosts including man, poultry and animals such as cattle, piglets, kids, foals, lambs and buffaloes Paul *et al*. (2010). Bacteraemia can rapidly lead to death or infection in different organs such as in the meninges, joints or eyes. In addition to economic losses, diarrhea in livestock is important because of the public health implications Tevejo *et al*. (2005). To be

able to trace reliably the source and mode of transmission of an outbreak, an epidemiological typing method must be able to discriminate reproducibly between different strains and identify identical strains. Virulence in microorganisms is associated with the capacity to attach and colonize at the site of infection, with subsequent damage to the host and is promoted by aggressins that interfere with the host defense Burrows (1985). Various studies indicate that pathogenic *E.coli* cause diarrheal diseases either by invasion of intestinal mucosa after attachment to host epithelial cells through pili or by elaboration of enterotoxins Giannella (1976).

It could be reported that virulent *E.coli* strains carried alpha hemolysin determinants and suggested that hemolysins might play a role in the pathogenicity of *E.coli* by releasing iron required for bacterial growth, by killing the host defense cells, or by there cytotoxic effects on kidney cells Cavalieri *et al.* (1984). Most strains of *E.coli* are harmless, saprophytes, however, some are pathogenic by virtue of plasmid mediated virulence factors through which disease is induced Janke *et al.* (1990). The quantitative estimation of Mg^{+2}activated ATP-ase determined in *E.coli* suspension through determining the amount of phosphate released colorimetrically (Butling *et al.*, 1973; raw, 1975; Helal *et al.*, 2011 and Helal *et al.*, 2013). Also previous trials of quantitative cytochemical determination of succinic dehydrogenase (SDH-ase) enzyme activity in *Bacillus megaterium* through determining the optical density of the end reaction product (formazan pigment) spectrophotometrically Hess and Dietrich, (1960). A recent trial for identification different strains of *Brucella* organisms compared to other bacterial species (*Salmonella Dublin*, *E.coli* O_{157} H_7 and *Plesiomonas shigelliodes*) through quantitative cytochemical estimation of ATP-ase and SDH-ase enzyme activities in their suspensions (with fixed concentration of $5X10^6$ CFU/ml). The study could obtained significantly different optical densities for each of the two enzymes (after one hour incubations with their specific substrates) and there were a reverse relationship between the calculated actual enzyme activities of the two enzymes Helal *et al.* (2011). Also another recent semular trial for cytochemical characterization of ATP-ase and SDH-ase enzymes for identification of bacteria causing mastitis Helal *et al.* (2013). The objective of this work is to isolate and identify some *E.coli* serogroups associated with buffalo calf diarrhea by traditional bacteriological methods with investigation of some important virulence factors associated with such isolates and used such isolates for additional cytochemical enzyme characterization for some bacterial enzymes as first trial for identification and differentiation between the serogroups of same species (*E.coli*) that causing enteritis in buffalo calves.

MATERIALS and METHODS

1- Collection of samples:
A total of 150 fecal samples were collected from buffalo calves 3 to 6 weeks of age (100 were diarrheic and 50 were non diarrheic).

These buffaloes were obtained from private farms in Cairo and Giza governorates. Aliquots of 5g of rectal feces were separately collected using sterile disposable plastic gloves that were inverted after sampling. Samples were transferred to the laboratory in a cold chamber container to be cultured as soon as possible.

2- Isolation and identification of *E.coli* strains:
Fecal samples were primarily cultured on MacConkey agar medium, incubated aerobically at 37ºC. After an overnight incubation, apart of single typical well isolated lactose fermenting colony was tested for sorbitol fermentation by culturing on sorbitol MacConkey agar and sorbitol phenol red agar media, then incubated at 37ºC overnight. Morphological, cultural and biochemical examinations were carried out according to Quinn *et al.* (1994).

3- Serological identification of *E.coli* isolates:
Antisera of *E.coli* were used for serological identification of somatic antigen 'O' using slide agglutination test according to Koneman *et al.* (1997). The *E.coli* immune-O-sera (polyvalent sera), 8 vials and Monovalent sera, 43 vials also, were obtained from Denka Seiken Co. LTD, Tokyo, Japan.

4-Detection of virulence factors of *E.coli* isolates:

4.1. Hemolytic activity (Hemolysin): was tested using 5% defibrinated sheep blood agar Beutin *et al.* (1989).

4.2. Enterotoxin (STa) detection: The ability to produce heat stable enterotoxin was assayed by the infant mouse test Robins-Brown *et al.* (1993).

4.3. Invasiveness assay: The ability of *E.coli* isolates to invade epithelial cells were tested Janda and Abbott, (1998).

4.4. Verotoxin activity of *E.coli* serovars: Detection of cytotoxin activity of *E.coli* strains isolated from the fecal samples using Vero cells Giugliano *et al.* (1982).

4.5. Congo red binding test: It´s a test which differentiate between pathogenic and non pathogenic *E.coli* isolates Berkhoff and Vinal, (1986).

5. Cytochemical identification of E.coli serogroups:

5.1. Preparation of bacterial suspension of 5X10^6 CFU/ml concentration: Using aseptic techniques, a single colony from each of the isolated and previously identified *E.coli* serogroup was transformed into 100ml bottle of Iso-Sensitest broth incubated overnight at 35ºC, centrifugated at 4000 rpm for 5 minutes to obtain clean sample of bacterial suspension, concentrated bacterial suspensions were prepared in saline with optical density of 500nm, from which serial dilutions with saline were prepared until the optical density become in range of 0.750nm, the actual number of colony forming units was calculated from the viability graph, the dilution factor needed was calculated and the dilution was carried out to obtain a concentration of bacterial suspension of 5X10^6 CFU/ml Sarker *et al*. (2007).

5.2. Preparation of substrate incubation media for Adenosine triphosphatase (ATP-ase) enzyme (Mg^{+2}-activated, lead method):
According toWachstein and Meisel, (1957).

5.3. Preparation of succinate substrate medium for succinate dehydrogenase SDH-ase enzyme activity:
According to Nachlas *et al*. (1957).

5.4. Procedure for qautitative estimation of bacterial ATP-ase (Mg^{+2}-activated, lead method): Wachstein and Meisel, (1957):

5.4.1**.** In five clean, dry and sterile test tubes, put 1ml of bacterial suspension of certain *E.coli* serogroup of bacterial concentration of 5X10^6 CFU/ml.

5.4.2. Add1ml of substrate incubation medium for ATP-ase enzyme in each tube containing bacterial suspension, gentle mixing and recording the optical density (OD.) at wave length (wl) of 555nm before incubation.

5.4.3. Incubate the mixture at 37ºC for exactly 30 minutes, add 0.5ml of 1% ammonium sulfide solution (the color then become dark brown).

5.4.4. Dilute the mixture with 4.5ml normal saline with gentle mixing and directly record the OD. of the mixture spectrophotometrically at 555nm.wl according to Andreu *et al*. (1973).

5.4.5. The above procedure was repeated for each of the rest serogroups of *E.coli.*

5.5. Procedure for quantitative estimation of bacterial SDH-ase enzyme activity: Nachlas *et al.* (1957): The same procedures for ATP-ase estimation was followed for SHD-ase enzyme estimation in the five *E.coli* serogroups, except in case of SDH-ase, there was no dilution and no chemical added and the OD. was read at WL of 450nm.

5.6. The semi-quantitative cytochemical determination of ATP-ase and SDH-ase enzyme reactions in different *E.coli* serogroups: Put 0.7ml from specific substrate medium for each enzyme with the same volume of bacterial suspension (from each of *E.coli* serogroup) on clean dry and sterile glass slide, incubated at 37ºC for 45 minutes. The SDH-ase reaction slides were counter-stained with 2% methylene blue, but the slides with ATP-ase reactions were added to it 4drops of 1% ammonium.

Sulfide and then counterstained with safranin-O (1%). All sides were dried and mounted with glycerol jelly and covered with cover slides to be examined microscopically with high power for ATP-ase or SDH-ase enzyme reactions semi-quantitatively which ranged from trace to intense enzyme reactions according to the intensity of diformazan pigment or lead sulfide pigment for SDH-ase and ATP-ase enzyme reaction respectively according to Pearse (1972).

5.7. Statistical Analysis:
The optical denisities obtained from the enzymes reactions of the different *E.coli* serogroups were statistically evaluated using the Analysis of variance (ANOVA) according to Snedecor and Cochran, (1969).

RESULTS

Table 1: Percentage of *E.coli* from buffalo calves fecal samples.

Source of isolates	No. of examined samples	No. of *E.coli* isolates	%
Apparently healthy buffalo calves	50	13	26
Diarrheic buffalo calves	100	72	72
Total	150	85	56.66

Table 2: *E.coli* serogroupes isolated from apparently healthy buffalo calves.

Isolates	O_{26}	O_{111}	O_{78}	O_{55}	O_{86}
No.	5	3	2	2	1
%	38.46	23.07	15.38	15.38	7.69

The percentage of *E.coli* isolates in relation to apparently healthy buffalo calves (13)

Table 3: *E.coli* serogroupes isolated from diarrheic buffalo calves.

Isolates	O_{26}	O_{111}	O_{78}	O_{55}	O_{86}
No.	30	15	14	7	6
%	41.66	20.83	19.44	9.72	8.33

The percentage of *E.coli* isolates in relation to diarrheic buffalo calves (72).

Table 4: Virulence determinants of *E.coli* strains isolated from apparently healthy and diarrheic buffalo calves fecal samples.

Virulence Factors	**Isolates from apparently healthy buffalo calves**					**Isolates from diarrheic buffalo calves**					**Total**	
	O_{26} (5)	O_{111} (3)	O_{78} (2)	O_{55} (2)	O_{86} (1)	O_{26} (30)	O_{111} (15)	O_{78} (14)	O_{55} (7)	O_{86} (6)	**No.**	**%**
Haemolytic activity	1	1	0	0	0	20	11	10	6	3	52	61.17
Enterotoxin Production	0	0	0	0	0	0	8	10	5	3	26	30.58
Verotoxin Production	1	0	1	1	0	20	0	10	5	3	41	48.23
Invasiveness	1	1	0	0	0	20	8	10	5	3	48	56.47
Congo red binding	2	1	1	1	1	20	11	10	6	3	56	65.88

The percentage was calculated in relation to the total number of the isolated *E.coli* strains (85)

The cytochemical study

A- The ATP-ase enzyme activity of different *E.coli* serogroups:

1- The optical densities (OD.) of ATP-ase before incubation period:
There were significant variations of the OD. of ATP-ase activitie between all *E.coli* serogroups.

2- The optical densities (OD.) of ATP-ase after incubation period:

There were no significant variation of the OD. of ATP-ase activities between *E.coli* serogroups (except between *E.coli* O_{55} and *E.coli* O_{111} which could be significantly differentiated from the other three groups) the highest ATP-ase activity showed by *E.coli* O_{55} (Table 5).

B-The SDH-ase enzyme activity of different *E.coli* serogroups:

1-The optical densities (OD.) of SDH-ase before incubation period:

There were significant variations of the OD. of SDH-ase activities between all *E.coli* serogroups.

2-The optical densities (OD.) of SDH-ase after incubation period:
There were significant variations of the OD. of SDH-ase activities between different *E.coli* serogroups except *E.coli* O_{26} and *E.coli* O_{111} which could not differentiated between each other as they showed the same OD. value. The highest SDH-ase activity was showed by *E.coli* O_{26}, but the lowest SDH-ase activity was showed by *E.coli* O_{86} (Table 6).

C- The Actual Enzymatic Reactions of ATP-ase and SDH-ase of *E.coli* serogroups:

The actual enzyme reactions is the difference between the OD. of enzyme activity after and before the incubation period.

1- Actual ATP-ase enzyme reaction:
The actual ATP-ase enzyme activities of the different *E.coli* serogroups which arranged from highest to lowest reactions are 285,265,255,205 and 160nm for *E.coli* O_{86},O_{78},O_{111},O_{26} and O_{55}respectively (Table 7).

2- Actual SDH-ase enzyme reaction:
The actual SDH–ase enzyme reactions of the different *E.coli* serogroups which arranged from highest to lowest reactions are 210,200,190,180 and 150nm for *E.coli*O_{111},O_{78}, O_{86},O_{26} and O_{55}respectively (Table 7).

3- The Reverse Relationships between the percentages of the actual ATP-ase and SDH-ase reaction:
There is a reverse relationship between the percentages of the actual ATP-ase and the actual SDH-ase enzyme reactions of *E.coli* serogroups (in relation to their higher actual enzyme reactions). Such reverse reactions between the percentages of the two enzymes of the different serogroups are recorded in Table (9) and illustrated in Fig. (1).

D- The semi-quantitative estimation of ATP-ase and SDH-ase enzyme reactions in different *E.coli* serogroups:

1- ATP-ase activity:
ATP-ase enzyme activities of the different *E.coli* serogroups ranged from moderate reactions (+++) in *E.coli*O_{55} to (strong to intense) reactions (++++±) in *E.coli*O_{111} (Table10 and Fig. 2-A and 2-B).

2- SDH-ase activity:
The SDH-ase enzyme reactions of the different *E.coli* serogroups ranged from submoderate reactions (++) in *E.coli*O_{86} to strong reactions (++++) in *E.coli*O_{26} and *E.coli*O_{111} (Table 10 and Fig. 2-C and 2-D).

Table 5: The quantitative estimation of Adenosine Tri-phosphatase (ATP-ase) bacterial enzyme activity of the different bacterial suspensions of the different serogroups (the concentration of the all bacterial suspensions =5X10^6 CFU/ml at optical density (OD.) of 0.750nm and WL=0.750nm).

E.coli Serogroups	*E.coli* O_{26}	*E.coli* O_{55}	*E.coli* O_{78}	*E.coli* O_{86}	*E.coli* O_{111}	LSD (at P≤ 0.005)
OD. of ATP-ase **before** incubation	0.390^{a} ± 0.002	0.405^{b} ± 0.001	0.325^{c} ± 0.002	0.305^{d} ± 0.001	0.345^{e} ± 0.001	0.003
OD. of ATP-ase **after** incubation	0.595^{ac} ± 0.001	0.565^{b} ± 0.019	0.590^{a} ± 0.002	0.390^{a} ± 0.002	0.600^{c} ± 0.002	0.010

N.B.: the different litters in rows denote presence of significant differences between means (at P≤ 0.005).

Table 6: The quantitative estimation of Succinate Dehydrogenase (SDH-ase) bacterial enzyme activities of the different *E.coli* serogroups bacterial suspensions (the concentration of the all bacterial suspensions =5X10^6 CFU/ml at optical density (OD.) of 0.750nm and WL=0.750nm).

E.coli Serogroups	*E.coli* O_{26}	*E.coli* O_{55}	*E.coli* O_{78}	*E.coli* O_{86}	*E.coli* O_{111}	LSD (at P≤ 0.005)
OD. of SDH –ase **before** incubation	0.380^{a} ± 0.002	0.400^{b} ± 0.002	0.340^{c} ± 0.001	0.300^{d} ± 0.002	0.350^{e} ± 0.002	0.002
OD. of SDH –ase **after** incubation	0.560^{a} ± 0.002	0.550^{b} ± 0.002	0.540^{c} ± 0.002	0.490^{d} ± 0.002	0.560^{a} ± 0.002	0.003

N.B.: the different litters in rows denote presence of significant differences between means (at P≤ 0.005).

Table 7: The actual reactions of ATP-ase and SDH-ase of the different *E.coli* serogroups (as difference between the OD. after and before (A-B) incubations and the values of actual reactions arranged from higher values).

E.coli serogroups	*E.coli* O_{86}	*E.coli* O_{78}	*E.coli* O_{111}	*E.coli* O_{26}	*E.coli* O_{55}
Actual ATP-ase reaction	285	265	255	205	160
E.coli serogroups	*E.coli* O_{111}	*E.coli* O_{78}	*E.coli* O_{86}	*E.coli* O_{26}	*E.coli* O_{55}
Actual of SDH-ase reaction	210	200	190	180	150

Table 8: The reverse relationships between the percentages of the actual ATP-ase and SDH-ase enzymes reactions of the *E.coli* serogroups in relation to the higher actual enzyme activities.

E.coli serogroups / Actual enzyme reactions	*E.coli* O_{86}	*E.coli* O_{78}	*E.coli* O_{111}	*E.coli* O_{26}	*E.coli* O_{55}
ATP-ase %	100	92.98	89.47	71.93	56.14
SDH-ase %	90.48	95.24	100	85.71	71.43

Table 9: The semi-quantitative estimation of ATP-ase and SDH-ase enzyme activities of different serogroups of *E.coli*.

*E.coli*serogroups / Actual enzyme reactions	*E.coli* O_{26}	*E.coli* O_{55}	*E.coli* O_{78}	*E.coli* O_{86}	*E.coli* O_{111}
ATP-ase %	++++	+++	+++±	+++±	++++±
SDH-ase %	++++	+++±	+++	++	++++

N.B. ±=traces of enzyme activity,+=week activity,++=submoderate activity,+++=moderate activity,++++=strong activity,+++++=intense enzyme activity.

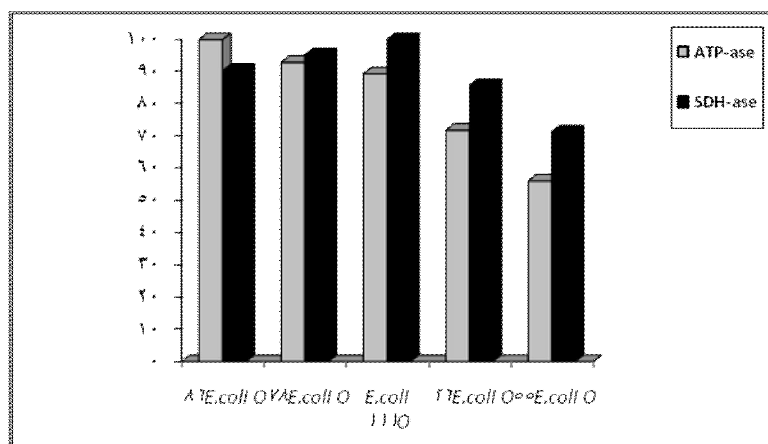
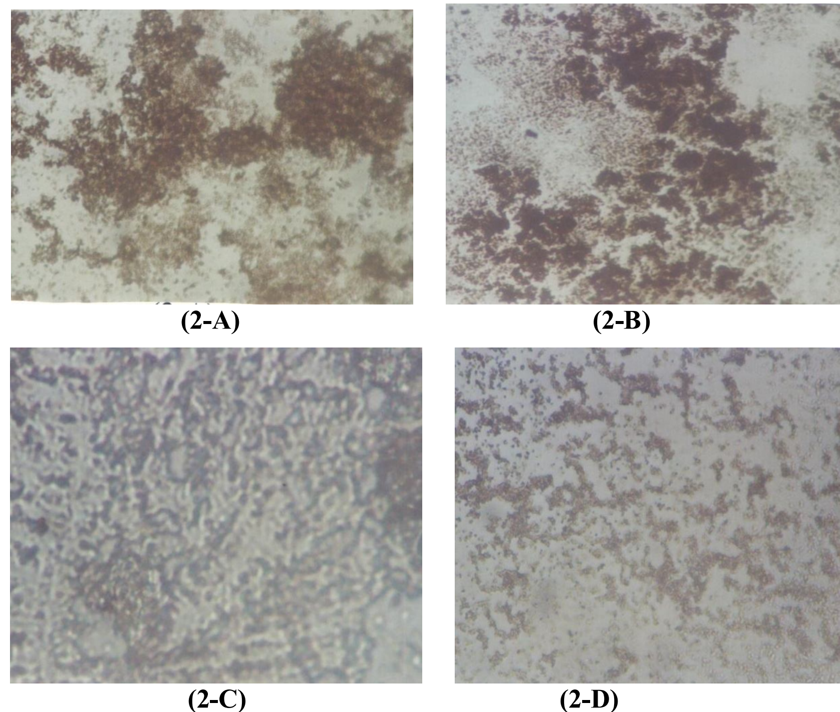


Fig. 1: The reverse relationships between the percentages of the actual reactions of ATP-ase and SDH-ase enzymes of the *E.coli* serogroups in relation to the higher actual enzyme activities.



(2-A) (2-B)

(2-C) (2-D)

Fig. (2): Showing the activities of ATP-ase and SDH-ase enzymes of the *E.coli* serogroups after incubation with their substrate media as following:
(2-A): strong tointense (+++++) ATP-ase enzymes activity of the *E.coli* O_{111}
(2-B): Moderate (+++) ATP-ase enzymes activity of the *E.coli* $O_{55,}$
(2-C): strong (++++) SDH-ase enzymes activity of the *E.coli* $O_{26,}$
(2-D): Sub-moderate (++) SDH-ase enzymes activity of the *E.coli* O_{86}

DISCUSSION

The role of *Escherichia coli* as a pathogen is well known and many *E.coli* isolates have been associated with a wide variety of diseases in animals. It´s incriminated in production of severe infections such as gastrointestinal colibacillosis, colisepticaemia, hemorrhagic colitis, haemolytic uraemic syndrome, bloody diarrhea and also it is a frequent cause of bovine mastitis. Therefore, increasing attention is being given to the role played by livestock in the epidemiology of this organism and to study biochemical, serological identification and virulence factors in addition to the enzyme cytochemical characterizations. In the present work, from 150 apparently healthy and diarrheic buffalo calves fecal samples 85(56.66%) were positive for *E.coli*. In addition, the recovery rates of *E.coli* isolated from apparently healthy and diarrheic buffalo calves fecal samples were 13 out of 50 (26%) and72 out of 100 (72%) respectively (Table1). These results confirmed that mentioned by Wells *et al.* (1991) and Karmali *et al.* (1985) who mentioned that such *E.coli* strains are mostly and frequently isolated from fecal samples of diarrheic calves. Traditional studies for *E.coli* were always clarified by referring to the serotyping of the incriminated isolates Orskov and Orskov, (1978). Serogrouping of *E.coli* was carried out according to the available antisera, 13 serogroups of *E.coli* were isolated from apparently healthy buffalo calves O_{26}(38.46%), O_{111}(23.07%), O_{78}(15.38%), O_{55}(15.38%) and O_{86}(7.69%) (Table 2); while 72 serogroups of *E.coli* were isolated from diarrheic buffalo calves O_{26}(41.66%), O_{111}(20.83%), O_{78}(19.44%), O_{55} (9.72%) and O_{86}(8.33%) (Table 3). Table 2 and 3 showed that from apparently healthy and diarrheic buffalo calvesO_{26} serogroup had the highest incidence (38.46% and 41.66% respectively), followed by O_{111} (23.07% and 20.83% respectively). In the same time, O_{26} and O_{111} showed nearly the same SDH-ase enzyme activities, as showed by the current cytochemical study and this need further clarification. Many studies have shown that O_{26} is the most clinically important genotype within the shigatoxin producing *E.coli* strains isolated from diarrheic and even non diarrheic cases because of its association with bloody and non bloody diarrhea and HUS (Hemolytic ureamic syndrome) Elliott *et al.* (2001) and Rivas *et al.* (2006). The virulence factors are thought to play an important role in diseases caused by *E.coli*, thus the virulence factors of the isolated *E.coli* serogroups were evaluated in this study.

An overall looking for the present study serotyping of *E.coli* isolated from apparently healthy and diarrheic buffalo calves fecal isolates as well as a trial to detect the virulence factors aiming to find some

characteristics that could be depended upon in identifying pathogenic *E.coli* strains that incorporated in calves diarrhea. In (Table 4), it´s clear that 52 isolates were hemolytic with percentage of 61.17% and belonged to serogroups O_{26}(21), O_{111}(12), O_{78}(10), O_{55} (6) and O_{86}(3) a result coordinated with Raji *et al*. (2003). As well as 26 isolates were positive for enterotoxin production with percentage of 30.58% belonged to serogroups O_{111} (8),O_{78} (10),O_{55} (5)and O_{86} (3), enterotoxigenic *E.coli* colonize the intestine by means of different host Specific Colonization Factors (SCF) and produce one or both of two enterotoxins, the heat stable (ST) and heat labile (LT) toxins which are both able to cause diarrhea Sjoling *et al*. (2006). While 41 isolates were positive for verotoxin production with percentage of 48.23% belonged to serogroupsO_{26} (21),O_{78} (11),O_{55} (6) and O_{86} (3). The relatively low incidence of non-O_{157} verotoxin production alone may not be sufficient for verotoxigenic *E.coli* to cause disease Barrett *et al.* (1992). Moreover 48 isolates were positive for invasiveness with percentage of 56.47% belonged to serogroupsO_{26} (21), O_{111}(9), O_{78}(10), O_{55} (6) and O_{86} (3), Donnenberg *et al.* (1989) used HEP-2 cell gentamicin invasion assay to compare the relative invasive ability of enteropathogenic *E.coli* to enterotoxigenic, enteroinvasive and verocytotoxic *E.coli*. Finally 56 isolates were positive for Congo red binding activity with percentage of 65.88% belonged to serogroupsO_{26} (22), O_{111} (12), O_{78}(11), O_{55} (7) and O_{86} (4), many workers successfully used Congo red binding assay in tryptose soya agar for identification of pathogenic *E.coli* Roy *et al.* (2006). By the world health organization as enteropathogenic *E.coli* or the classical enteropathogenic *E.coli* were: O_{26} ,$O_{55,}$ O_{86} ,O_{111} ,O_{114} ,O_{119} ,O_{125} ,O_{126} ,O_{127} ,O_{128} ,O_{142} and O_{158} Hernandes *et al.* (2009). From the all previous results, there were several methods for bacterial identification of the different bacterial species and serotypes, such as biochemical methods Cowan and Steel, (1974), serological Oda *et al.* (1979), morphological Bailey and Scott, (1990), polymerase chain reaction (PCR) for nucleic acid identification Sambrook *et al.* (1989). The cytochemical method which should considered as one branch of biochemical method as a recently developing method that firstly described for the purpose of bacterial identification by Rostagi *et al.* (1984). They could clearly separate the *Mycobacterium leprae* from certain leprosy derived Corynform bacteria qualitatively. The present study used such cytochemical method through the quantitative determination of some bacterial enzymes in bacterial suspensions with a fixed concentration for the identification or differentiation of the different serogroups of *E.coli* organisms. The estimation of bacterial ATP-ase and SDH-ase enzyme activities in these serogroups was carried out under the all fixed factors of either cytochemical or bacteriological techniques. Previous trails for quantification of bacterial ATP-ase enzyme activities in *E.coli* suspension by Bulting *et al.* (1973). Recently by our trails for differentiation of the different species of Brucella organisms compared with other bacterial species Helal *et al.* (2011) and more recently for cytochemical differentiation between different strains infecting mammary gland in buffaloes and cattle Helal *et al.* (2013) through quantitative determination of bacterial ATP-ase and SDH-ase enzyme activities. The quantitative cytochemical enzyme determination were carried out spectrophotometrically or calorimetrically as previously reported by Buttling *et al.* (1973) and raw, (1975). For the purpose of quantitative cytochemical determination of bacterial enzyme activity in the different bacterial strains, we should fix all cytochemical and bacteriological.

Factors as: culture age before preparing the bacterial suspensions and fresh substrate media for the enzyme reactions (within 3 weeks maximum). For more benefits, the cytochemical method should performed on certain groups of bacteria infecting different body systems, such as bacteria causing enteritis, bacteria causing mastitis, bacteria causing respiratory infections or different serotypes of certain species of bacteria for their close relations in these cytochemical trials. In the previous trial Helal *et al.* (2011) the bacterial concentration in suspensions of the different bacterial strains was $15x10^8$ CFU/ml using turbidity standard of McFarland method Quinn *et al.* (1994) and the incubation period of bacterial suspension with their enzyme-substrate media was 1 hr., but in the present study the concentration of bacterial suspension was $5x10^6$CFU/ml using spectrophotometer at wave length of 0.750nm according to Sarker *et al.* (2007) for the purpose of accuracy of quantitative estimation, also the incubation period was ½ hr. for saving time. As soon as (within 30 seconds) from adding substrate medium of the enzyme to the bacterial suspension we record the optical density (OD.) of the mixture which was of great importance to recognition of the occurrence of enzymatic reaction after the period of incubation, where the OD. elevated significantly. If no significant elevation of the second OD. reading after incubation, this pointing to failure of enzyme reaction due to presence of significant technical error. The current cytochemical study revealed that the bacterial ATP-ase activity in the different *E.coli* serogroups showed non-significant changes between the all serogroups except between *E.coli* O_{55}serogroup of lower ATP-ase activity which could then differentiated it from other studied *E.coli* serogroups. The Entrohemorrhagic *E.coli* (EHEC) is the most pathogenic *E.coli* group which produce shiga-like toxin and cause bloody diarrhea with little or no fever, and if the disease left untreated, it may lead to hemorrhagic colitis as caused by O_{26},O_{111},O_{86} and O_{78}, while O_{55} which is an enteropathogenic *E.coli* (EPEC) cause watery diarrhea Dobrowsky *et al.*

(2013). This indicated that the serogroup *E.coli*O_{55} is less pathogenic than that of the other studied *E.coli* serogroups: O_{26},O_{78},O_{86} and O_{111} which of higher ATP-ase than O_{55}serogroup as there was direct relationship between the bacterial virulence and ATP-ase enzyme activity. Such virulence property that correlated with higher ATP-ase could be suggested by previous work Helal *et al.* (2013) in bacteria causing mastitis, where the higher ATP-ase reaction was detected in the more virulent bacteria causing mastitis and vice versa. The ATP-ase enzymes of bacteria include a diverse range of proteins (as pom-z protein) that involved in spatial regulation of bacterium such regulator used for identification of certain types of bacteria Monanhan and Harry, (2012). The significantly similar ATP-ase activity between most *E.coli* serogroups need another cytochemical assays for their differentiation as SDH-ase enzyme (discussed later), lactic dehydrogenase, glucose-6-phosphate gehydrogenase or NADH-diaphorase enzymes determinations for completing figure of enzyme characterizations of the different *E.coli* serotypes Sheehan and Hrapchak, (1980). Concerning to the succinic dehydrogenase (SDH-ase) enzyme activity, the current study revealed presence of significant variations between SDH-ase enzyme activities among the different *E.coli* serogroups after incubation period (except between *E.coli* o_{26} and *E.coli* O_{111} which showed the same SDH-ase activity). The SDH-ase is the only enzyme of the Kreb's cycle that could convert succinate to fumarate Altschal *et al.* (1996) could use a semi-quantitative cytochemical method for determination of the oxidative enzyme cytochrome-c-oxidase for identification of bacterial species. For more identification and characterization of the different *E.coli* serogroups by the cytochemical study, the actual enzyme activity (the difference between OD. after and before incubation which calculated for ATP-ase and SDH-ase enzymes) should be considered. When we arranged the actual ATP-ase reactions from the higher to lower values and then obtaining the percentage of each value (in relation to the higher value) we could obtain different five percentages from 100% to 56.14% for the ATP-ase actual reactions and when we compared such percentages of ATP-ase with the corresponding percentages of SDH-ase of the same bacterial serogroup (Table 8) we could obtain a reversible relationship between ATP-ase and SDH-ase actual enzyme reactions (Fig.2). Such reversible reactions between the two enzymes were carried out in bacterial mitochondrial membranes as the lack of ATP-ase molecules (due to the activation of ATP-ase enzyme) and the excess of oxaloacetate molecules leading to the suppression of SDH-ase enzyme reactions in bacterial and plant cells indicating the reversible relationships between ATP-ase and SDH-ase in bacteria (Hung *et al*., 2010, Helal *et al*., 2011 and Helal *et al*., 2013). Based on the present data, the ATP-ase activity could not differentiate between the different *E.coli* serogroups (except between the serogroup *E.coli* O_{55} of the significant lower ATP-ase ($P \leq 0.05$) and that of other species, but it could.

Be differentiate between the majority of *E.coli* serogroups by the significant difference of SDH-ase activity in *E.coli* serogroups (except between the serogroups *E.coli* O_{26} and *E.coli* O_{111} which were showed the same SDH-ase activity). Additionally, the values of actual ATP-ase and SDH-ase reactions could be used for differentiation between *E.coli* serogroups, so that four values for each *E.coli* serogroup could be used for its characterization and identification are: (OD. of ATP-ase& actual.

ATP-ase reaction and OD. of SDH-ase and actual SDH-ase reaction) as an additional tool for bacterial differentiation and identification of *E.coli* serogroups.

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استخدام الطرق التقليدية والسيتوكيميائية الكمية للتعرف على بعض العترات السيرولوجية للميكروب القولوني المسببة للالتهابات المعوية في الجاموس والتوصيف الإنزيمي لها

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يعتبر الإسهال من أكثر أمراض الجاموس خطورة خاصة في العجول الرضيعة والصغيرة ، ومن اهم البكتريا المصاحبة لهذا المرض هو الميكروب القولوني (الايشيرشيا كولاي) وهي تشمل العديد من العترات السيرولوجية وتم اختبار العترات المعزولة مع المحتوى الإنزيمي لهذه الإنزيمات لمقارنة الطرق التقليدية بالمستويات الانزيمية لها ، لذلك تم الحصول على ١٥٠ عينة براز (١٠٠من عجول جاموس مصابة بالإسهال و٥٠ من عجول غير مصابة بالإسهال (سليمة اكلينكيا) تم تجميعها من مزارع خاصة لعزل ميكروب الايشيرشيا كولاي للتعرف على العترات المختلفة من هذا الميكروب باستخدام الطرق المورفولوجية والبيوكيميائية والسيرولوجية بالإضافة للطرق السيتوكيميائية للقياس الكمي لمستويات بعض الإنزيمات للميتوكوندريا البكتيرية (الادينوزين ثلاثي الفوسفاتيز والسكسينات ديهيدروجينيز (ATP-ase & SDH-ase) لتقييم الفروق المحتملة بين الإنزيمين بين العترات المختلفة من الميكروب القولوني (*E.coli*-serogroupes). وأوضحت النتائج السيتوكيميائية انه توجد ٤ مقادير إنزيمية لكل عترة (اثنين لكل انزيم) بناءا على هذا النموذج (الكثافة الضوئية لإنزيم ATP-ase، والتفاعل الحقيقي للإنزيم & الكثافة الضوئية لإنزيم SDH-ase، والتفاعل الحقيقي للإنزيم) للعترات السيرولوجية للميكروب *E.coli* التالية: *E.coli*O_{26} ,*E.coli* O_{55}, *E.coli* O_{78}, *E.coli* O_{86},*E.coli*$_{111}$ كالتالي:(0.595،0.285& 0.560،0.210)،(0.550, 0.200, 0.265 & 0.565) ، (0.540, 0.190, 0.255 & 0.590) ، (0.490, 0.180, 0.205 & 0.590)، (0.560, 0.150, 0.160 & 0.600) / nm على التوالي. وبناء على هذه الدراسة فقد امكن استنتاج أن انزيم السكسينات ديهيدروجينيز SDH-ase يمكن بمستوياته المختلفة ان يفرق بين العترات السيرولوجية المختلفة لميكروب الايشيريا كولاي (ماعدا عترتي *E.coli*O_{26} and *E.coli*O_{111}) اللتين أظهرتا تشابه في مستوى الانزيم، بينما أظهر مستوى انزيم الادينوزين ثلاثي الفوسفات ATP-aseعدم القدرة على التفريق بين مختلف عترات الميكروب القولوني (ماعدا عترة *E.coli*O_{55}) التي قل مستوى الإنزيم بها عن بقية العترات الأربعة الأخرى معنوية، ولهذا يمكن أن يستخدم مستوى التفاعل الحقيقي (Actual enzyme activity) وهو الفرق بين الكثافيتين الضوئيين قبل وبعد فترة تحضين الإنزيم البكتيري مع المادة المتفاعلة معه الوسط الكيميائي الخاص به، وينصح ايضا بعمل دراسات تكميلية سيتوكيميائية على إنزيمات بكتيرية أخرى لاستكمال التوصيف الإنزيمي لجميع عترات الميكروب القولوني والتي يمكن بها أن تفرق بين العترات المختلفة لهذا الميكروب كطريقة أخرى للتعرف على أنواع البكتريا المختلفة.