Phenotypic and Genotypic characterization of *Edwardsiell tarda* isolated from *Oreochromis niloticus* and *Clarias gariepinus* at Sohag Governorate

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Abstract: ***Edwardsiella*** *tarda* is a common fish pathogen, it causes one of the most significant septicemic diseases responsible for mass mortality in freshwater fishes and consequently high economic losses. This study was carried out to investigate prevalence of *E. tarda* among *O. niloticus* (Nile tilapia) and *C. gariepinus* (African catfish) at Sohag Governorate and to characterize the isolates phenotypically and genotypically in addition to detection of Major fimbrial subunit gene (*etfA*) in them by PCR assay. Therefore, 93 samples of *O. niloticus* and 87 samples of *C. gariepinus* collected from different localities at Sohag Governorate during the period from March 2014 to March 2015. Fish samples were subjected to clinical and post-mortem examination then bacteriological examination for liver, kidney and spleen. The suspected isolates were characterized by cultural and morphological characters, some conventional biochemical tests and API 20E system then by PCR assay. 9 isolates were characterized as *E. tarda* [4 isolates (E1-E4) from *O. niloticus* (4.3%) and 5 isolates (E5-E9) from *C. gariepinus* (5.7%)]. The phenotypic characterization of the isolates revealed that they were homogenous except in citrate utilization and similarity percentage between them was ranged from 96.3 to 100%. Furthermore, Major fimbrial subunit gene (*etfA*) was demonstrated in all *E. tarda* isolates by PCR. Results of this study indicated that polymerase chain reaction is very reliable and rapid method for identification of *E. tarda* isolates which may be helpful in prevention and control of Edwardsiellosis.

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Keywords: *Edwardsiell tarda*, *Oreochromis niloticus*, *Clarias gariepinus*, PCR.

1. Introduction

Fish play an important role as a cheap, palatable and highly nutritive source of animal protein **(Othman *et al*., 1993)**. World production of fish has steadily increased to meet the growing global demand **(Saleh *et al*., 2009)**. In Egypt, majority of fish farms can be classified as semi-intensive earthen pond farms, *O. niloticus* and *C. gariepinus* are the two main species reared in these farms (**Ibrahem *et al*., 2011**).

Bacteria are the most prevalent cause of morbidity and mortality among wild populations of fish and they cause major losses in freshwater aquaculture anywhere **(Mohanty and Sahoo, 2007)**. *Enterobacteriaceae* considered the most important bacteria that cause severe economic losses among fish farms all over the world **(El-Gamal and Saad, 2009)**. *E. tarda* causes edwardsiellosis in freshwater and marine fishes of both farmed and wild population allover the world **(El-Jakee *et al*., 2008)**. Edwardsiellosis is one of the most important bacterial diseases in fish and it causes mass mortality in the various populations and age groups of fish.

E. tarda is a Gram-negative, short, rod-shaped bacterium of about 1 µm in diameter and 2-3 µm in length **(Evans *et al*., 2011)**. There are two phenotypic strains of *E. tarda*; typical and atypical **(Yasunaga *et al*., 1982)** and based on O-agglutination test, there are four serotypes of *E. tarda*; A, B, C and D **(Park *et al*., 1983)**. *E. tarda* strains isolated from various geographical sources exhibit little variation in the phenotypic characters **(Austin and Austin, 2007)** and several studies have demonstrated a wide degree of intraspecific diversity in the isolates from the different geographic regions and host species **(Nucci *et al*., 2002 and Wang *et al*., 2011).**

It has been shown that different strains, serotypes, genotypes and biotypes of bacterial pathogens vary in their ability to cause disease within aquaculture (**Austin *et al*., 2003)**, so accurate identification and characterisation of a pathogen is of paramount importance for both control and epidemiological investigations **(Tinsley *et al*., 2010)**. Also, it is well known that diagnosis of a particular infection depends on detection and identification of its causative agent **(Das *et al*., 2014)**.

Pathogenesis of *E. tarda* is multifactorial and several potential virulence factors contribute in it have been reported **(Mohanty and Sahoo, 2007)**. Several

reports found that fimbrial adhesin-like protein, motility-related proteins as flagellin and autotransport adhesin AIDA were important for attachment and penetration of *E. tarda* into the host epithelial cells **(Sakai *et al*., 2009)**. Detection of type I fimbrial gene of *E. tarda* by PCR is important factor to confirm both identification and pathogenicity of *E. tarda* isolates **(Sakai *et al*., 2007 and Korni *et al*., 2012)**.

E. tarda were isolated from freshwater fishes in some regions in Egypt (**Mosad *et al*., 1992; Saad El-Deen *et al*., 2005; Mahmoud *et al*., 2006; Ali *et al*., 2008 and Ramadan *et al*., 2009)** but for our knowledge, only one study was carried on *E. tarda* in *C. gariepinus* at Sohag Governorate by **Hashiem and Abd El-Galil (2012)**.

Considering the above facts, the present study was carried out to investigate prevalence of *E. tarda* among the diseased *O. niloticus* and *C. gariepinus* at Sohag governorate, to isolate and identify *E. tarda* phenotypically and genotypically and to detect Major fimbrial subunit gene (*etfA*) in the isolates by PCR technique.

2. Material and Methods

Fish samples and clinical examination

A total of 93 samples of diseased *O. niloticus* and 87 samples of diseased *C. gariepinus* were collected from different localities at Sohag Governorate during the period from March 2014 to March 2015, these samples included live fishes, moribund and recently dead ones.

The collected fishes were transported rapidly to the laboratory in insulated ice box containing ice or commercial cool packs. They were subjected to clinical and post-mortem examination according to **Amlacher (1970)** for determination and recording the clinical abnormalities present externally and internally.

Isolation of *E. tarda*

After disinfection the outer surface of the skin with ethyl alcohol 70%, samples for bacteriological examination were collected by a sterile bacteriological loop from the internal organs (liver, kidney and spleen) under aseptic condition. The collected samples were immediately inoculated into Brain Heart Infusion Broth (LAB, England) and incubated under aerobic condition at 35°C for 24 hours then plated on *Salmonella-Shigella* agar (Oxoid, England) and incubated at 35°C for 24 hours **(Muratori *et al.*, 2001)**. The isolates were preserved frozen at -20°C in Tryptone Soya Broth (Oxoid, England) supplemented with 20% glycerol till further use **(Maiti *et al.*, 2009)**.

Phenotypic characterization of *E. tarda*

This was done on basis of cultural and morphological characters, some conventional biochemical tests and API 20E system (bioMerieux, France) according to manufacturer's instructions. The isolate was identified as *E. tarda* according to criteria of **Wyatt *et al.* (1979)** and **Fang *et al.* (2006)**.

Genotypic characterization of *E. tarda* and detection of Major fimbrial subunit gene (*etfA*)

DNA was extracted from the isolates using DNeasy® Blood and Tissue extraction kit (QIAGEN, Valencia, CA) according to the manufacturer's protocol, its concentration was measured by spectrophotometer then was preserved at -20°C or immediately used.

DNA was amplified using GoTaq® Hot Start Green Master Mix (Promega, USA) and oligonucleotide primers target 415 bp fragment of gyrB gene of *E. tarda* (F gyrB1 5`GCATGGAGACCTTCAGCAAT3` and R gyrB1 5`GCGGAGATTTTGCTCTTCTT3`) and Major fimbrial subunit gene (*etfA*) of *E. tarda* (F 5`CGGTAAAGTTGAGTTTACGGGTG3` and R 5`TGTAACCGTGTTGGCGTAAG3`) and in 25 µl reaction mixture according to mastermix manufacturer's instructions where contain 12.5 µl of GoTaq® Hot Start Green Master Mix, 0.6 µl from each of forward and reverse primers, 1–5 µl from extracted DNA and Nuclease-Free Water to complete the mixture 25 µl. PCR reaction was performed in a thermocycler under the conditions previously described by **Lan *et al.* (2008)** for 415 bp fragment of gyrB gene (30 cycle of denaturation at 94°C for 1 minute, annealing at 51.5°C for 30 second and extension at 72°C for 30 second followed by final extension at 72°C for 10 minutes) and those previously described by **Sakai *et al.* (2007)** for Major fimbrial subunit gene (*etfA*) (initial denaturation at 94°C for 2 minutes followed by 30 cycle of denaturation at 95°C for 20 second, annealing at 55°C for 20 second and Extension at 72°C for 1 minute followed by final extension at 72°C for 7 minutes).

Agarose gel electrophoresis was made for PCR products according to **Sambrook *et al.* (1989)** and the amplified DNA on the gel were observed and photographed. Isolate was considered positive as *E. tarda* and contain Major fimbrial subunit gene (*etfA*) when visible band appeared at 415bp.

3. Results

Results of clinical and post-mortem examination

Examination of *O. niloticus* infected with *E. tarda* revealed presence of abdominal distension, hemorrhages on the body surface, congestion of the fins, skin ulcers and congested vent. Internally, there were accumulation for the ascitic fluid and congestion of the internal organs including liver, spleen and kidney (photos 1, 2 and 3) while examination of *C. gariepinus* infected with *E. tarda* revealed presence of skin ulcerations, hemorrhages on the body surface, congestion of the fins and fin rot. Internally, there

were hemorrhages on the muscles, congestion and enlargement of liver, spleen and kidney, small white nodules were observed on surface of kidney and liver in some cases (photos 4, 5 and 6).

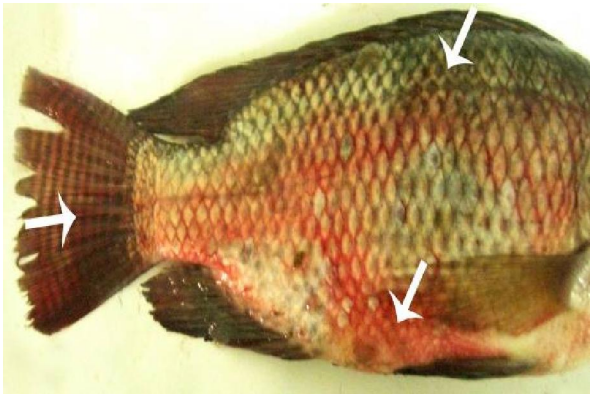

Photo (1): *O. niloticus* infected with *E. tarda* showing body darkening, hemorrhages on the skin and congestion of the fins.

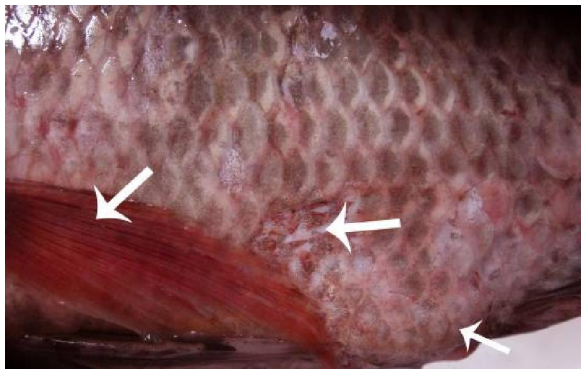

Photo (2): *O. niloticus* infected with *E. tarda* showing hemorrhages on the skin, scale loss and skin ulcers and congestion of pectoral fin.

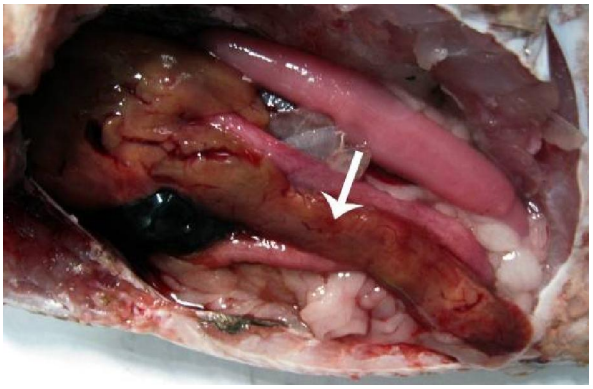

Photo (3): *O. niloticus* infected with *E. tarda* showing congested liver.

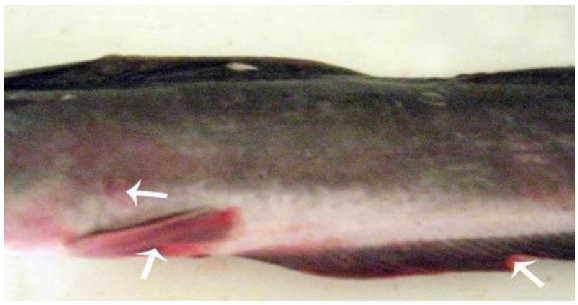

Photo (4): *C. gariepinus* infected with *E. tarda* showing hemorrhages on the skin and congestion of the fins.

Photo (5): *C. gariepinus* infected with *E. tarda* showing congestion of the liver and presence of small necrotic foci on its surface.

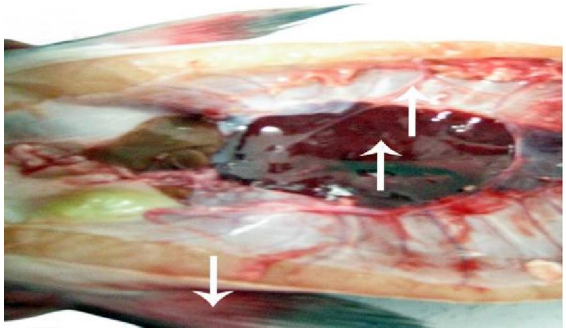

Photo (6): *C. gariepinus* infected with *E. tarda* showing highly congested kidney, presence of small necrotic foci on its surface and hyperemia.

Results of *E. tarda* isolation from the examined fishes

4 (E1-E4) and 5 (E5-E9) *E. tarda* isolates were isolated from the examined *O. niloticus* (4.3%) and *C. gariepinus* (5.7%) respectively.

Results of phenotypic characterization for *E. tarda* isolates

On *Salmonella-Shigella* agar, colonies of *E. tarda* were small transparent with black centers to entire black colonies. Microscopical examination for Gram-stained smears prepared from these colonies,

revealed Gram-ngative short bacilli. Also, microscopical examination for wet preparations from them revealed motile bacteria. Biochemical characterization of the isolates revealed that all the 9 isolates were biochemically homogeneous except in citrate utilization where all the isolates were positive in catalase, methyl red reduction, lysine decarboxylase, ornithine decarboxylase, fermentation of glucose, H_2S production & indole production and negative in cytochrome oxidase, lactose fermentation test, ß-galactosidase, arginine dihydrolase, urease, tryptophane deaminase, Voges-Proskauer, gelatinase and utilization of mannitol, inositol, sorbitol, rhaminose, sucrose, melibiose, amygdalin and arabinose. 7 *E. tarda* isolates (77.8%) were negative in citrate utilization test and 2 isolates (22.2%) were positive in such test (photos 7 and 8). It could be concluded that the isolates were typical strains of *E. tarda* and similarity percentage of their phenotypic characteristics was ranged from 96.3 to 100%.

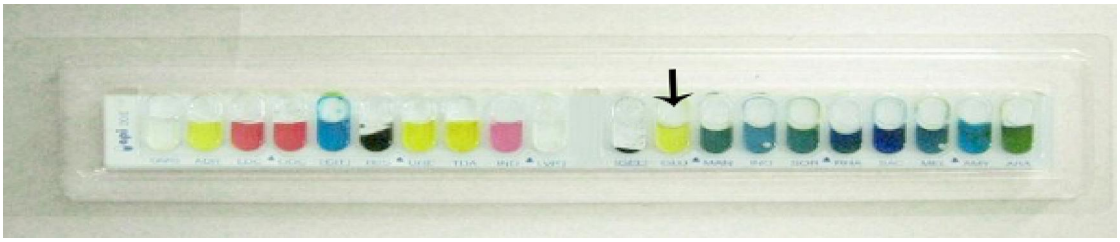

Photo (7): Characterization of *E. tarda* isolate by API 20E strip, the isolate was positive in citrate utilization test.

Photo (8): Characterization of *E. tarda* isolate by API 20E strip, the isolate was negative in citrate utilization test.

Results of Genotypic characterization for *E. tarda* isolates

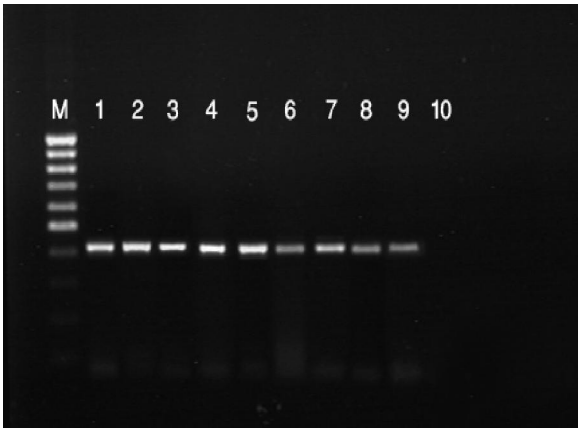

Photo (9): Agar gel electrophoresis of PCR products using specific primers targeting 415 bp fragment of gyrB gene in *E. tarda* isolates. Lane M: molecular weight marker 100 base pair; lanes 1-9: DNA extracted from *E. tarda* isolates (E1:E9 respectively) showing bands at 415 bp and lane 10: negative control.

By polymerase chain reaction using specific primers targeting a 415 bp fragment of gyrB gene of *E. tarda*, amplification of the extracted DNA from all the isolates which characterized as *E. tarda* by cultural, morphological and biochemical characteristics revealed bands at 415 bp. No bands were observed in negative control sample (photo 9).

Results of *E. tarda* isolates investigation for Major fimbrial subunit gene (*etfA*) by PCR

By polymerase chain reaction using specific primers targeting Major fimbrial subunit gene (*etfA*) of *E. tarda*, amplification of the extracted DNA from all *E. tarda* isolates revealed bands at approximately 415-bp and this indicated that the isolates contain fimbrial gene and their pathogenicity. No bands were observed in negative control sample (photo 10).

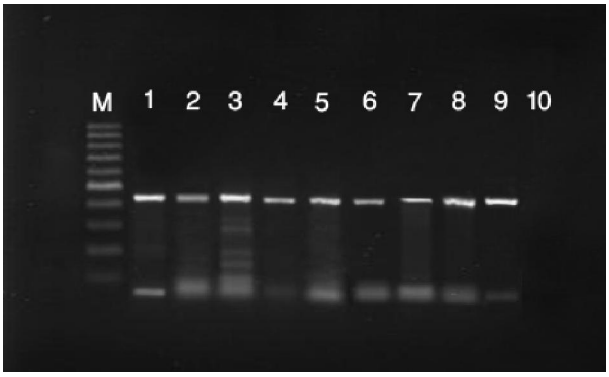

Photo (10): Agar gel electrophoresis of PCR products using specific primers targeting Major fimbrial subunit gene (*etfA*) in *E. tarda* isolates. Lane M: molecular weight marker 100 base pair; lanes 1-9: DNA extracted from *E. tarda* isolates (E1:E9 respectively) showing bands at 415 bp and lane 10: negative control.

4. Discussion

Edwardsiellosis is one of the most important bacterial diseases in fishes and it causes massive mortalities in the various populations and age groups of fish and consequently high economic losses **(Plumb, 1993 and Jun and Yin, 2006)**. Information about incidence of *E. tarda* is required to determine its significance in infection of fish and take the appropriate measures to control edwardsiellosis. It was found that prevalence of *E. tarda* infection among the examined *O. niloticus* was 4.3% and this result came in accordance with results of **Ali *et al.* (2008)** who reported that incidence of *E. tarda* among the diseased *O. niloticus* at Beni-Suef Governorate was 3.7%. Higher rate of *E. tarda* among *O. niloticus* was recorded by **Korni *et al.* (2012)** who recorded that prevalence of Edwardsiellosis among the cultured *O. niloticus* in spring season at Beni-Suef Governorate was 13.33 %. Our results also was disagreed with **Abd El-Mageed *et al.* (2002)** who recorded that incidence of *E. tarda* in *O. niloticus* collected from different localities in Egypt was 0%. The difference in prevalence of *E. tarda* may be attributed to the difference in water temperature, stocking density, water quality and/or location of the study.

While prevalence of *E. tarda* infection among the examined *C. gariepinus* was 5.7% and this result came in accordance with results of **Hashiem and Abd El-Galil (2012)** who reported that incidence of *E. tarda* among *C. gariepinus* at Sohag Governorate was 5.2%. Very higher rates of *E. tarda* among *C. gariepinus* were recorded by **Eissa and Yassien (1994)** and **Abd El-Mageed *et al.* (2002)** who reported that incidence of *E. tarda* among *C. gariepinus* was 24.93% and 27.7% respectively while lower rate was reported by **Ramadan *et al.* (2009)** who reported *E. tarda* in *C. gariepinus* at El Fayoum Governorate with an incidence of 0.42%. These differences may be attributed to the difference in water temperature, stocking density, water quality and/or location of the study.

Examination of *O. niloticus* infected with *E. tarda* revealed number of the clinical signs as abdominal distension, hemorrhages on the body surface, congestion of the fins, presence of skin ulcers and congested vent. Internally, there were accumulation for the ascitic fluid and congestion of internal organs including liver, spleen and kidney. These clinical signs and post-mortem lesions were similar to those reported by **El-Deeb *et al.* (2006)** and **Galal *et al.* (2002)**. **Kubota *et al.* (1981)** and **Korni *et al.* (2012)** reported additional clinical signs and post-mortem lesions in the naturally diseased *O. niloticus* as exophthalmia, opacity or hemorrhages of the eyes and presence of small white nodules in the gills, kidney, liver and spleen. These abnormalities weren't recorded in our study and this may be attributed to the difference in the fish immune state or the environmental conditions which determine severity of *E. tarda* septicaemia.

While examination of *C. gariepinus* infected with *E. tarda* revealed presence of skin ulcerations, some of these ulcers were severe reaching the muscles, hemorrhages on the body surface, congestion of the fins and fin rot. Internally, there were hemorrhages on the muscles, congestion and enlargement of liver, spleen and kidney and sometimes small white nodules were observed on surface kidney and liver. These clinical signs and post-mortem lesions were similar to those reported by **Eissa and Yassien (1994); Saad El-Deen *et al.* (2005); Ramadan *et al.* (2009); Yu *et al.* (2009)** and **Hashiem and Abd El-Galil (2012)**.

Biochemical characterizations have proved to be a valuable method for typing and differentiation of bacterial fish pathogens **(Austin *et al.*, 1997)**. Phenotyping is used in conjunction with serology and genotyping to identify and study the bacterial pathogens **(Coquet *et al.*, 2002)**. In this study, the isolates were characterized based on the morphological characters of the colonies, microscopical examination and biochemical tests then investigated by Polymerase Chain Reaction (PCR).

In this study, *E. tarda* suspected colonies on *Salmonella-Shigella* (SS) agar grown forming small transparent colonies with black centers to entire black one. This result was similar to findings of **Wyatt *et al.* (1979)** and **Xiao *et al.* (2009)**. By microscopical examination, the isolates were consists of Gram-ngative short bacilli and motile. These results came in agreement with results of **Baya *et al.* (1997)** and **El Deeb *et al.* (2006)** who isolated Gram-negative,

motile short bacilli of *E. tarda* from wild striped bass and different freshwater fishes (Nile tilapia, carp and catfish) respectively and to some extent with results of **Wyatt *et al.* (1979)** and **Lan *et al.* (2008)** who isolated non-motile *E. tarda* form catfish and turbot respectively.

With regard to the biochemical characteristics of *E. tarda* isolates, results of the conventional biochemical tests and API 20E revealed that all *E. tarda* isolates were biochemically homogeneous except in citrate utilization. All the isolates were cytochrome oxidase negative, positive in catalase, methyl red reduction, lysine decarboxylase, ornithine decarboxylase, fermentation of glucose, H_2S production, and indole production while negative in ß-galactosidase, arginine dihydrolase, urease, tryptophane deaminase, Voges-Proskauer, gelatinase, and utilization of Lactose, mannitol, inositol, sorbitol, rhaminose, sucrose, melibiose, amygdalin and arabinose while 77.8% of them were negative in citrate utilization test and 22.2% were positive.

The biochemical characteristics of *E. tarda* isolates in this study were in agreement with results of **Wyatt *et al.* (1979)** and **Fang *et al.* (2006)** except in case of citrate utilization test results. The variation among *E. tarda* isolates in their ability to utilize citrate as a sole source of carbon and energy for growth was reported by **Korni *et al.* (2012)** who found that 11.8% of *E. tarda* isolates failed in citrate utilization and by **Castro *et al.* (2006), Acharya *et al.* (2007)**, **Maiti *et al.* (2009), Park *et al.* (2012)** and **Das *et al.* (2014)**. In contrast, **El Deeb *et al.* (2006)** and **Saad El-Deen *et al.* (2005)** found that *E. tarda* isolates were homogenous and citrate-negative while **Hashiem and Abd El-Galil (2012)** and **Ibraheem *et al.* (2014)** found that all *E. tarda* isolates were citrate-positive.

In this study, all *E. tarda* isolates were homogenous and gave positive results in both indole and H_2S production tests, this agreed with results of **Xiao *et al.* (2009)** and **He *et al.* (2011)**. **Joh *et al.* (2010)** who reported that some *E. tarda* isolates were indole-negative (13.5%) and H_2S-negative (38.9). The variation in any phenotypic characteristic may be attributed to presence or absence of plasmid (s) that controls its metabolic trait. Indole production, H_2S production and citrate utilization tests are important biochemical characteristics for differentiation of *Enterobacteriaceae* members, furthermore, *E. tarda* and *E. ictaluri* can be differentiated biochemically through indole production and H_2S production tests in addition to methyl red reduction test. So, careful attention should be taken when identifying and differentiating of *E. tarda* using these biochemical characteristics.

The gyrB gene was regarded as a suitable phylogenetic marker for identification and classification of bacteria **(Yamamoto *et al.*, 1999)**. Comparison of gyrB gene of *E. tarda* with the phylogenetically related *Enterobacteriaceae* strains revealed high sequence divergence and in comparison with 16S rDNA sequences, *E. tarda* gyrB exhibited only 75-84% homology with the available gyrB gene sequences in *Enterobacteriaceae* contrary to 92–95% homology in the 16S rDNA sequences **(Lan *et al.*, 2008)**. In this study, DNA extracted from the phenotypically characterized *E. tarda* isolates were examined by polymerase chain reaction using a specific primer pair amplify 415 bp fragment of gyrB gene of *E. tarda*. Agar gel electrophoresis for PCR products revealed bands at approximately 415 bp from all the isolates as shown in photo 9 and this confirm the phenotypic identification of *E. tarda* isolates. These results agreed with findings of **Lan *et al.* (2008)** who observed the 415 bp fragment of gyrB gene in all their *E. tarda* isolates. Our findings indicated that identification of *E. tarda* by PCR using specific primers target 415 -bp fragment of gyrB gene is very reliable and rapid method in diagnosis of edwardsiellosis and this is helpful to its control and preventing epidemics occurrence and subsequently decreasing the economic losses which occur due to the delay in disease diagnosis and the rapid intervention.

To understand pathogenicity of *E. tarda* identification and characterization of the virulence factors are strongly needed **(Ali *et al.*, 2008)**. Improving background data on virulence factors of the pathogen from different areas facilitates development of reliable and useful diagnostics and vaccines for it. Detection of type I fimbrial gene of *E. tarda* by PCR is important factor to confirm both identification and pathogenicity of *E. tarda* isolates **(Sakai *et al.*, 2007 and Korni *et al.*, 2012)**. In the present study, Major fimbrial subunit gene (*etfA*) was detected in all the isolates which phenotypic and genotypic characterized as *E. tarda* as shown in photo (10) and this result confirm identification and pathogenicity of *E. tarda*. Detection of Major fimbrial subunit gene (*etfA*) by PCR can be used as a rapid and specific method for identifying pathogenic *E. tarda* isolates.

Conclusion

4 and 5 *E. tarda* isolates were isolated from *O. niloticus* and *C. gariepinus* at Sohag Governorate, these isolates were homogeneous except in citrate utilization test. Our findings indicated that identification of *E. tarda* by PCR is very reliable and rapid method in diagnosis of edwardsiellosis and this is helpful to its control and preventing epidemics occurrence and subsequently decreasing the economic losses.

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