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Aeromonas Infections in African Sharptooth Catfish

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Abstract

The aim of this study was to investigate the dominant species of *Aeromonas* in naturally infected African Sharptooth catfish, *Clarias gariepinus*, in Qena, Egypt and the distribution of two virulence genes among the isolates to assess their pathogenicity. Twenty-five isolates of *Aeromonas* were recovered from infected fish showing signs of septicemia. Restriction-fragment-length-polymorphism (RFLP) analysis of the 16S-rDNA amplified products demonstrated that the specie isolated were *Aeromonas hydrophila* (56%) and *Aeromonas veronii* (44%). Isolates were screened for the cytotoxic enterotoxin, *act*, and aerolysin, *aerA*, genes. The *act* gene was detected only in *A. hydrophila*, while the *aerA* gene was more frequently found among all isolates. Catfish challenged with an *A. hydrophila* isolate that have both the *act* and *aerA* genes showed higher mortalities (80.9%) and more severe signs of septicemia than those challenged with an isolate that lacks both genes studied.

Keywords: *Aeromonas*; *Clarias gariepinus*; *act* gene; *aerA* gene; RFLP analysis

Introduction

Several cases of septicemia in African Sharptooth catfish, *Clarias gariepinus*, have been submitted to our lab by the local fishermen in Qena, Egypt, who reported that such case, have recently increased in the small tributaries of the River Nile. Although there is no official data for the rates of infections and mortalities, our preliminary investigations indicated that main cause of such infections is *Aeromonas*. Genus *Aeromonas* causes serious problems in various fish and shellfish species that is characterized by septicemia and resulting in mass mortalities and high economic losses [1,2]. Out of the 24 reported species within the genus [3], only *Aeromonas hydrophila*, *Aeromonas veronii*, *Aeromonas caviae*, and *Aeromonas jandaei* are considered the main species that infect fish [4,5] causing Motile *Aeromonas* Septicemia (MAS).

There are many extracellular virulence proteins that contribute to the pathogenicity of *Aeromonas* spp. [6], including exotoxins such as haemolysins, cytotonic and cytotoxic enterotoxin [7,8] and aerolysin [9]. The cytotoxic enterotoxin (*act*) is aerolysin related with approximately 90% homology [10]. The *act* gene is a major virulence factor of *Aeromonas* that can create pores in the erythrocyte membranes, [11]. It possesses hemolytic, cytotoxic, and enterotoxic activities [12]. Aerolysin gene (*aerA*) is recorded to be the commonly regarded virulence gene produced by some strains of *Aeromonas*, so its detection proposed to be a reliable approach to investigate pathogenic *Aeromonas* strain. It is an extracellular, soluble, hydrophilic protein exhibiting both haemolytic and cytolytic properties [13,14] by pore formation, as it binds to eukaryotic cells and aggregates to creates unadjusted pores in the membrane of targeted cells leading to the destruction of the membrane permeability and lysis [15].

The precise and accurate identification and characterization of a pathogen, together with the detection of the prospect virulence traits are the corner stone for epidemiological investigations and accordingly designing the control programs and preventive measures. The current study aimed to investigate the species distribution of *Aeromonas* isolates among naturally infected African Sharptooth catfish in Qena, Egypt. The distribution of two extracellular virulence genes among the isolates was investigated as a means of assessing the pathogenicity to catfish based on the genetic profile. Additionally, a challenge study was conducted to further define the correlation between the genetic profiles of the isolates and their pathogenicity.

Materials and Methods

All experiments were done according to the recommendations listed in the care and use of fish in research, teaching and testing section in the guide to the care and use of experimental animals, Canadian Council on Animal Care (CCAC), Ottawa, Ontario, Canada [16].

Clinical examination and bacterial isolation

Fifty African Sharptooth catfish with average weight of 100 to 150g and total length of 23 to 27 cm showing signs of septicemia were submitted by the local fishermen to the Aquatic Diagnostic Laboratory, Faculty of Veterinary Medicine, South Valley University. Fish were caught from small tributaries of the River Nile at Qena Governorate, Egypt. Inoculations from the kidneys and spleen were made on tryptic soya broth, TSB (Oxoid, England), and incubated at 28°C for 24 hours. Then, the broth cultures were streaked on *Aeromonas* selective agar-base, ASA (Biolife, Italy) and incubated at 28°C for 24 hours, where green colonies with dark centers were presumptively considered to be *Aeromonas* [17].

Conventional identification of the suspected isolates

Conventional phenotypic identification was conducted according to Austin et al. [18] based on the morphological, biochemical and metabolic characters. It included Gram stain, oxidase, catalase, indole (Kovac´s method), voges-proskauer, methyl red, H_2S production, esculin hydrolysis, acid and gas production from glucose, motility using semisolid agar, growth on 6 and 10% sodium chloride, and resistance to 150 g/ml of vibriostatic agent 0/129 (Oxoid).

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Molecular identification of the suspected isolates to the genus level

Bacterial DNA was extracted from the suspected isolates using the Gene JET genomic DNA purification kit (Thermo Scientific, EU) according to the manufacturer recommendations and then kept at -20°C until the time of use Polymerase chain reactions (PCR) were conducted to amplify a *gyrB*-gene target of approximately 1100 base pair (bp) using *Aeromonas*-specific primers [19]. Amplicons were analysed using 1.5% agarose gel electrophoresis in Tris-acetate EDTA (TAE) buffer, stained with ethidium bromide (50 µl/L) and visualized on UV transilluminator system (MultiDoc- It, UVP, UK).

Molecular identification of the suspected isolates at the species level

Polymerase chain reactions were conducted to amplify a hypervariable segment of the *Aeromonas* 16S-rDNA of about 1500 bp using genus-specific primers as per Borrell et al. [20]. Amplicons were subjected to restriction-fragment-length-polymorphism (RFLP) analysis with *Bst*SNI and *Mbo*I restriction enzymes (Invitrogen, USA) as per Ghatak et al. [21], and the digestion products were analysed using 1.5% agarose gel electrophoresis as previously described.

Detection of virulence genes in the *Aeromonas* isolates

To investigate the distribution of two virulence genes, *act* and *aerA*, among the *Aeromonas* isolates, a PCR study was conducted using the primers listed in Table 1 and the protocol described by Hu et al. [22]. Amplicons were analysed using 1.5% agarose gel electrophoresis as previously described.

Pathogenicity of the *A. hydrophila* isolated to catfish

African Sharptooth catfish with average body weight of 100 ± 5 g and total length 25 ± 3 cm were obtained from a private fish farm with no history of *Aeromonas* septicemia and were acclimated for 2 weeks in fiber glass aquaria supplied with dechlorinated tap water and aeration, at the wet laboratory, Department of Fish Diseases, Faculty of Veterinary Medicine, South Valley University. Two strains, one strain with act^+aerA^+ and another one with act^-aerA^- genotypes were used in experimental infection of catfish. Acclimated catfish were divided into 4 groups with 7 fish each in a completely randomized design. The first group was intra-peritoneally (I/P) injected with 0.5 ml of 6×10^6 cfu/ml of act^+aerA^+ *A. hydrophila*. The second group was I/P injected with act^-aerA^- *A. hydrophila* with the same dose as above. The third group was I/P injected with 0.5 ml of sterile saline (sham control), and the fourth group was un-injected as a negative control. The entire study was done in three replicates where clinical signs, post mortem lesions, and mortalities were recorded daily for up to two weeks. Moribund catfish were bacteriologically examined to re-isolate the causative *Aeromonas* strain from the internal organs. Identification of re-isolated bacteria was conducted by the molecular approaches as described above.

Results

Clinical examination and bacterial isolation

Collected catfish showed the signs of bacterial septicemia that included exophthalmia, skin ulceration, abdominal distension, scattered haemorrhages on the body surface and muscles, congestion of the fins, and fin rot. Internally, there were accumulations of yellowish or bloody ascetic fluids with friable, congested and enlarged organs.

Conventional identification of the suspected isolates

Bacteriological examination of the catfish resulted in recovery of 25 isolates were suspected to be *Aeromonas* based on their morphology on the ASA. Based on the conventional phenotypic, morphologic and biochemical characterization of the isolates, all the 25 isolates were presumptively identified as *Aeromonas*, although they showed variable results for esculin hydrolysis and gas and H_2S production on TSI (Data not shown).

Molecular identification of the suspected isolates

Using the *gyrB* primers resulted in amplification of targets of all the 25 isolates giving amplicons of 1100 bp as shown in Figure 1. In addition, 16S-rDNA targets of the isolates were amplified and used for RFLP analysis. Restriction digestions of the 16S-rDNA amplicons with *Bst*SNI resulted in two patterns of digestions, where only 14 (56%) amplicons were digested giving two fragments of 1104 and 462 bp length as shown in Figure 2 and their corresponding isolates were identified as *A. hydrophila*, while the other 11 (44%) amplicons remained uncut. Digesting these remaining 11 amplicons with *Mbo*I resulted in five fragments of about 618, 445, 219, 160 bp, and a smaller fragment of less than a 100 bp in length as shown in Figure 3 and their corresponding isolates were identified as *A. veronii*.

Detection of virulence genes in the *Aeromonas* isolates

Primers targeting the cytotoxic enterotoxin (*act*) and aerolysin (*aerA*) genes resulted in amplicons of 232bp and 301bp respectively, (Figures 4 and 5), and revealed that the act gene was found only in *A. hydrophila* with rate of 2/14 (14.3%) isolates (Table 2) while aerolysin (*aerA*) gene was present in 17/25 (68%) isolates, out of which 9 isolates

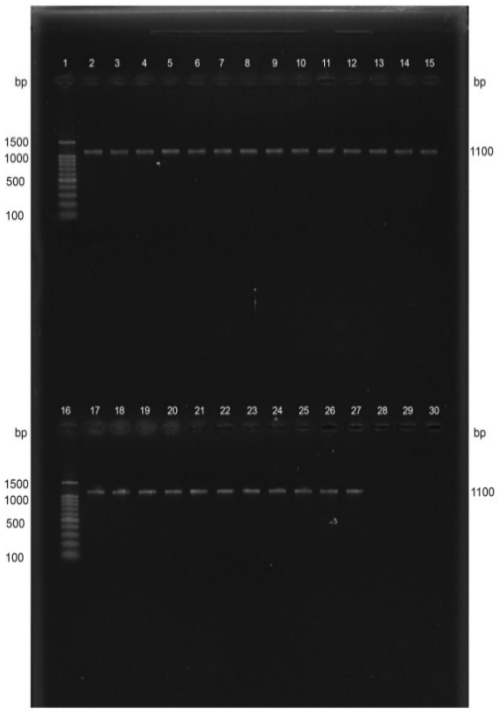

Figure 1: Amplifying a 1100 bp fragment of *gyrB*-gene of clinical isolates of *Aeromonas* using *Aeromonas*-specific primers. Lane 1 and 16: 100 bp ladder, Lane 2-15 and 17- 27: Clinical isolates of the present study, Lane 29: Negative control (No DNA).

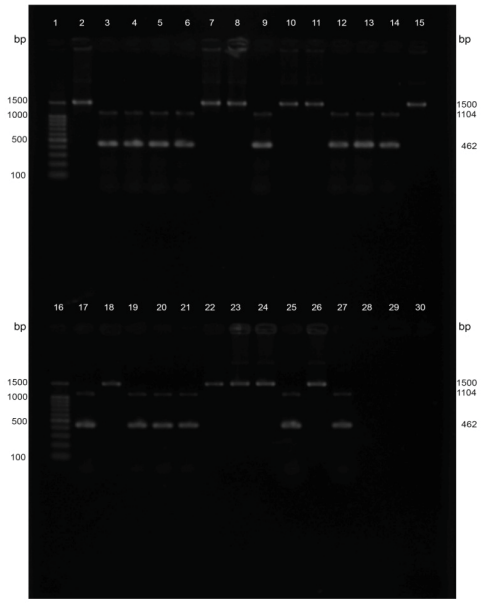

Figure 2: Restriction digestion of 25 *Aeromonas* spp. 16S-rDNA amplicons (1500 bp) with *Bst*SNI restriction enzyme. Lane 1 and 16: 100 bp ladder, Lanes 3-6, 9, 12-14,17, 19-21, 25, and 27: Digested *Aeromonas hydrophila* showing two fragments of 1104 and 462 bp length. Other *Aeromonas* spp. amplicons (1500 bp) were uncut (Lanes 2, 7, 8, 10, 11, 15, 18, 22-24 and 26).

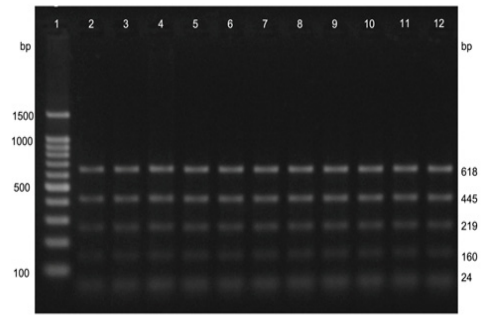

Figure 3: Restriction digestion of *Aeromonas veronii* 16S-rDNA amplicons (1500 bp) with *Mbo*I restriction enzyme. Lane 1: 100 bp ladder; lanes 2-12: Digested *A. veronii* showing 618, 445, 219, 160 and less than 100 bp fragments.

were *A. hydrophila* with rate of 9/14 (64.3%), (Table 2). Based on the distribution of the two genes, the 25 isolates were classified into four genotypic groups as act^+aerA^+ (2/25, 8%), act^+aerA^- (0/25, 0%), act^-aerA^+ (15/25, 60%), and act^-aerA^- (8/25, 32%).

Pathogenicity of the *A. hydrophila* isolated to catfish

The *A. hydrophila* isolate with the act^+aerA^+ genotype caused 80.9% average mortalities of the fish challenged. The clinical signs and post-mortem findings seen on fish challenged included scattered hemorrhages allover different parts of the body, sloughing and congestion of the caudal fin, and hemorrhages and deep skin ulcers at the caudal peduncle, and severe abdominal distention. Post mortem examination revealed the presence of severe inflammatory response with hemorrhages in the abdominal cavity, hemorrhages and congestions in ovaries, liver, kidney and spleen, while other fish showed pale coloration of the liver. Challenging the catfish with the same dose

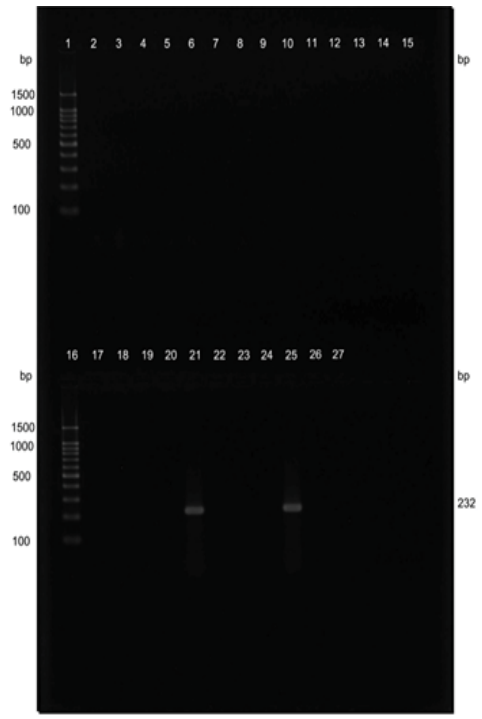

Figure 4: Detection of enterotoxin (*act*) gene in 25 *Aeromonas* spp. isolates showing a 232 bp amplicons in positive strains (Lanes 21 and 25). Lanes 1 and 16 are 100 bp ladder.

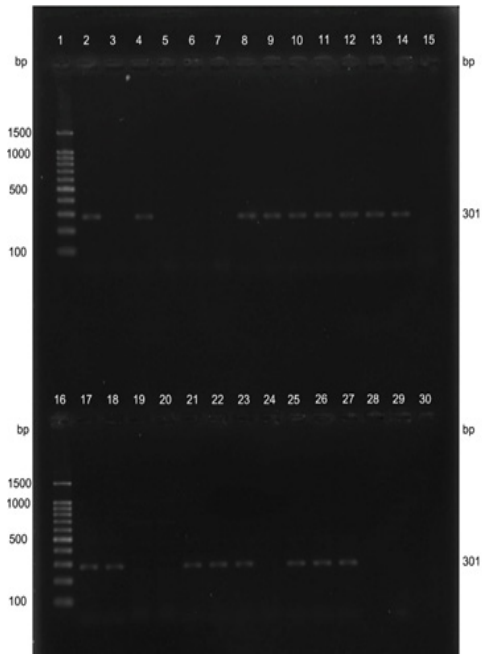

Figure 5: Detection of aerolysin (*aerA*) gene in 25 *Aeromonas* spp. isolates showing a 301 bp amplicons in positive strains (Lanes 2, 4, 8-14, 17, 18, 21-23 and 25-27). Lanes 1 and 16 are 100 bp ladder.

of the other *A. hydrophila* isolate (act^-aerA^- genotype) resulted in only 54% average mortalities where the clinical signs and lesions seen were less severe than those observed with the other isolate (act^+aerA^+).

Target genes	Primer sequence	Product sizes/bp	Reference
gyrB	F 5´ TCCGGCGGTCTGCACGGCGT 3 ´	1100	[19]
	R 5´ TTGTCCGGGTTGTACTCGTC 3´		
16S-rDNA	F 5' AGAGTTTGATCATGGCTCA 3'	1502	[20]
	R 5' GGTTACCTTGTTACGACTT 3'		
act gene	F 5´ GAGAAGGTGACCACCAAGAAC 3´	232	[22]
	R 5´ AACTGACATCGGCCTTGAACTC 3´		
aerA gene	F 5´ AACCGAACTCTCCAT 3´	301	[22]
	R 5´ TTGTCCGGGTTGTACTCGTC 3´		

Table 1: Primers used in the current study.

Genes	*A. hydrophila* (n=14)	*A. veronii* (n=11)	Total (n=25)
act gene	2 (14.3%)	0 (0%)	2 (8%)
aerA gene	9 (64.3%)	8 (72.7%)	17 (68%)

Table 2: The distribution of *act* and *aerA* virulence genes in *Aeromonas hydrophila* and *Aeromonas veronii* isolates.

Discussion

The distribution of *Aeromonas* isolates among infected African Sharptooth catfish was studied and their pathogenicity to catfish based on the genetic profile was assessed to investigate natural infection incidences reported with losses in the catfish in small tributaries of the River Nile at Qena governorate, Egypt. Fish pathogenic aeromonads are ubiquitous inhabitants of the aquatic ecosystems [23] making their interactions with fish uncontrolled [24]. Motile aeromonads are not uncommon in wild fishes. It was reported that gizzard shad (*Dorosoma cepedianum*) of Potomac River (Maryland, USA) had motile *Aeromonas* septicemia because of spawning stress [25]. Also, Paniagua et al. [26], were able to recover *Aeromonas* isolates from the River Porma, Leon, Spain that were identified as *A. hydrophila*, *A. sobria* and *A. caviae*.

To investigate the dominant pathogenic *Aeromonas* spp. in Sharptooth catfish in this study, accurate and definitive identification of the isolates is essential. Correct identification of the pathogen is crucial for the epidemiological studies, tracing-back disease outbreaks, and designing the appropriate control programs and treatment. In the present study a combination of conventional morphologic and metabolic characters was used to presumptively identify 25 isolates as *Aeromonas* spp. but was not conclusive in identifying the isolates to the species level. The genus identity of the isolates was, however, confirmed by amplifying the housekeeping gene, *gyrB*, using *Aeromonas*-specific primers [19]. All the 25 isolates were accurately identified to the species level as *A. hydrophila* and *A. veronii* by RFLP of the 16S-rDNA gene using a combination of two restriction enzymes. RFLP has been shown to be suitable for routine laboratory practices giving more easily recognizable DNA-band patterns to differentially identify the clinically important *Aeromonas* to the species level [20,27]. The minor deviations observed in the sizes of the digested fragments in case of *A. veronii* from those reported by Ghatak et al. [21] may be due to strain differences of the bacteria isolated in the present study from those previously investigated [21].

Aeromonas hydrophila and *A. veronii* were not only the dominant, but in fact the only aeromonads isolated from catfish in the present study. *A. hydrophila* is one of the predominant aeromonads in fish [28] and has been associated with great fish mortalities around the world [3]. Also, previous studies showed that *A. hydrophila* and *A. veronii* were the most prevalent aeromonad species found in fish and water [4,24]. In another study, *A. veronii* was the most common species isolated from fish and water environment, while *A. hydrophila* isolates were significantly more frequent in diseased fish than in healthy ones [22]. On the other hand, *A. sobria* was the dominant *Aeromonas* isolated from diseased fish in Spain [29].

The pathogenicity of *Aeromonas* spp. can be evaluated using the virulence determinants as genetic indicators [30]. In the present study, PCR assays have been used for the detection of two major *Aeromonas* virulence determinants (*act* and *aerA*) to assess the pathogenicity of the isolates. The cytotoxic enterotoxin gene, *act*, is one of the primary genes that makes *Aeromonas* pathogenic [31]. Also, the presence of hemolytic gene aerolysin, *aerA*, is an irrefutable indication of virulence in pathogenic *A. hydrophila* [32,33].

In this study, 25 *Aeromonas* isolates were classified into four genetic groups by defining their respective *act* and *aerA* genes (act^+aerA^+, act^+aerA^-, act^-aerA^+, and act^-aerA^-). Interestingly, the *act* gene was detected only in *A. hydrophila* isolates of the present study and was less frequent (14.3%) than in other reports where *act* was the most frequently found enterotoxin gene [34]. Furthermore, 65% of *Aeromonas* strains out of 350 clinical and environmental isolates were positive for *act*/*hlyA*/*aerA* [30]. Also, a wide variety in the combinations of virulence factors were reported in the *Aeromonas* isolates [34]. On the other hand, the distribution of the *aerA* gene among the *Aeromonas* isolates of the present study was higher (64.3%) than that of the *act* gene. Generally, *aerA* is widely distributed among *Aeromonas* isolates [35,36].

Virulence is essentially related to disease and pathology and subsequently should be evaluated in terms of morbidity and mortality of the host, thus *in-vivo* challenge studies is crucial to investigate pathogenicity [37]. In this study, as *A. hydrophila* was the dominant *Aeromonas* isolated, and thus was used in experimental infection of catfish. Challenging catfish with the *A. hydrophila* strain which has both of virulence genes (act^+aerA^+) caused higher mortalities and severe clinical signs when compared to those caused by the less virulent strain (act^-aerA^-). The *act* gene has the ability to lyse red blood cells by creating pores in the erythrocyte membranes [11,38]. This may explain the external and internal haemorrhages with septicemia seen on catfish challenged in the present study. In addition, the *act* gene has cytotoxic as well as tissue damage activities which affected the liver, kidneys, and other internal organs in the challenged catfish, herein, making them congested and friable. Furthermore, the ascites noticed in the present study may be due to activation of proinflammatory cytokines by the *act* [39]. On the other hand, the hemolytic and cytolytic activities of the aerolysin gene [40] found in some strains of *A. hydrophila*, may explain the bloody ascites and internal tissue damage associated with the challenge of catfish in the present study.

Interestingly, *A. hydrophila* strain that lacks the two virulence genes (act^-aerA^-) produced less mortalities (54%) and less severe signs than those recorded with the virulent strain (act^+aerA^+). Previous studies reported that an *act*-isogenic mutant was significantly attenuated in a mouse model [39], and the 50% lethal dose of *act* mutants in mice was 1.0×10^8, compared to 3.0×10^5 for the wild-type *Aeromonas*, where reintegration of the wild type *act* gene in these mutants resulted in complete restoration of the virulence in mice. Similarly, inactivation of the *aerA* gene resulted in a nine-fold increase in LD_{50} in the suckling mouse model [40].

Although the *act* and *aerA* genes are major virulence determinants of *Aeromonas*, they are not the only virulence genes reported to contribute to the pathogenicity during infections. This was supported

by the mortalities and signs seen in the present study associated with the strain that lacks both *act* and *aerA* genes. Virulence of *Aeromonas* is complex and not necessarily because of a particular virulence gene but likely requires the interaction of several virulence genes [36]. Synergy between virulence determinants may occur, where the *act* gene in *A. hydrophila* is iron regulated [39] that could be unregulated by the action of the *aerA* gene releasing iron from haemolyzed RBCs.

Conclusion

The present study clearly shows that *A. hydrophila* is the dominant *Aeromonas* infecting catfish in Qena, Egypt. *A. hydrophila* harbor many virulence factors where *act* and *aerA* genes play a major role in inducing lesions and diseases in catfish.

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