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Short communication

Shigatoxigenic and atypical enteropathogenic *Escherichia coli* in fish for human consumption

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ABSTRACT

Shigatoxigenic and enteropathogenic *Escherichia coli* with virulence and multidrug resistance profile were isolated from Nile tilapia. This study finding is of great importance to public health because they help understand this pathogen epidemiology in fish and demonstrate how these animals can transmit *E. coli* related diseases to humans.

Escherichia coli (*E. coli*) is not a natural inhabitant of the fish microbiota, nevertheless, it can be isolated from these animals gut due to its presence in contaminated aquatic environments.[1] It is worth noticing that this microorganism have pathogenic strains standing out as emerging zoonotic potential, as well as shigatoxigenic (STEC) and enteropathogenic (EPEC) *E. coli*. STEC strains produce the shiga toxin (Stx), which is its main virulence factor. There are two classes of shiga toxin, Stx1 and Stx2, with the last one presenting seven subtypes.[2] The EPEC may be either typical or atypical, with the atypical strains do not carrying virulence factor that encodes the bundle-forming pilus (bfp), but it carries the *eae* gene, that is located at the locus of enterocyte effacement (LEE), which is a pathogenicity island, that promote attaching and effacing lesions (A/E). The ability to induce A/E lesions is mediated by genes located on the LEE, as well as additional ones that are outside of it.[3]

Several studies have analyzed STEC and EPEC, and their virulence in humans,[4] cattle,[5] sheep,[6] pigs,[7] and buffaloes.[8] However, only a few studies have analyzed the presence of STEC and EPEC in fish[9,10] and, of these, none has detected presence of adhesion and ESBL genes. In addition, none has performed the *stx2* subtyping in STEC strains from fish. In this regard, this pioneer study aimed to compare the prevalence

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of STEC and EPEC strains in intensively farmed fish and free-living fish; as well as to detect their virulence and antibiotic resistant profile and analyze their genetic similarity looking for how these fishes contribute to humans infections.

The Ethics Committee on Animal Use (CEUA) approved this study under the protocol number 04076/14. Primers used are described in Table 1. The samples were collected from the fish species *Oreochromis niloticus*, from six different fish farms and three ranches located at northeast region of Sao Paulo state. A total of 472 samples were collected. Three hundred and seventy three (373) samples were obtained from fish farm animals and of these, 275 were from stools, 80 from muscles and 18 from the nurseries water. The other 99 samples were obtained from free-living fish, these been 90 from stools and nine from the river water. Samples were transferred to tubes containing BHI broth (Brain Heart Infusion) and after an incubation period, the DNA were extracted by thermal lysis according to Borges.[7]

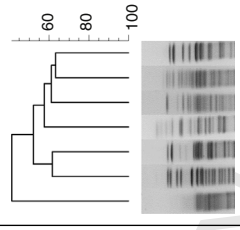
Screening for the detection of STEC and EPEC strain were based on the, *stx*1, *stx*2 and *eae* genes detection by multiplex PCR.[7] When one of these genes were detected, individual colonies from each sample were tested by PCR to isolate STEC and EPEC strains according to the protocol available at www.apzec.ca/en/APZEC/Protocols/pdfs/ECL_PCR_Protocol.pdf. This methodology is in accordance to the OIE Reference Laboratory for *Escherichia coli* (EcL – Faculté de Médecine Véterinaire, Université de Montréal). Isolates were further submitted to another PCR to detect others virulence genes as follow: *bfp*A, *ehx*A, *saa*, *iha*, *tox*B, *efa*1, lpfA$_{O113}$, *lpf*A$_{O157/OI-141}$, *lpf*A$_{O157/OI-154}$, *ast*A and *paa* genes. The Stx2 variants analysis was performed by *stx*2 subtyping according to Scheutz.[2]

The antimicrobial susceptibility test was performed using the disc diffusion method.[30] The antimicrobials chosen were the ones most used in fish farming and which are important for the detection of resistance genes dissemination. In this regard, drugs tested were ampicillin (10 μg), cephalothin (30 μg), streptomycin (10 μg), gentamicin (10 μg), ciprofloxacin (5 μg), chloramphenicol (30 μg), tetracycline (30 μg), nitrofurantoin (300 μg), nalidixic acid (30 μg), sulfamethoxazole and trimethoprim (25 μg), ceftriaxone (30 μg), cefoxitin (30 μg), kanamycin (30 μg), norfloxacin (10 μg), enrofloxacin (5 μg) (Oxoid). In addition, *E. coli* isolates were screened for extended-spectrum beta-lactamase (ESBL) genes for the *bla*$_{CTX-M}$ genotype groups 1, 2, 8, 9 and 25, the *bla*$_{TEM}$, and the *bla*$_{SHV}$.[11]

Phylogenetic *E. coli* groups' classification was performed according to the methodology proposed by Clermont.[12] Serotyping was performed at the *E. coli* Reference Center (EcRc) at Pennsylvania State University. The O somatic antigen were determinate by agglutination plates, also the PCR-RFLP of *fliC* gene, which encodes flagella, were performed to determine the H flagella antigen. Somatic antigens used were O1 to O187, with the exception of O31, O47, O67, O72, O94, O122 and the flagellar antigens used were H1 to H49, except H17, since these serogroups still not have been designated.

The isolates were also characterized by PFGE pattern of the PulseNet protocol as described by Ribot.[13] Briefly, the chromosomal DNA was digested with Xba1 and the electrophoresis conditions were an initial time of 2.2 s and an end time of 54.2 s in a gradient of 6V and the gels were electrophoresed for 21 h. The fragment similarities were compared using the Dice coefficient and the dendrogram was constructed by neighbor-joining grouping using BioNumerics software (Applied Maths, Sint-Martens-Latem, Belgium). MLST was performed following the Achtmans's scheme (http://mlst.ucc.ie/mlst/dbs/Ecoli), through the sequencing of the PCR amplification products of the *adk*, *fum*C, *gyr*B, *icd*, *mdh*, *pur*A, and *rec*A genes. The generated sequences were trimmed and analyzed by the Phred/Phrap/Consed software package.

All the results are shown in Fig. 1. Of the 373 analyzed samples from the fish farm, one (0.2%), from stools, tested positive for a STEC related gene (isolate 125F5). Of the 99 free-living fish analyzed samples, six (6%), also from stools, were positive for at least one of the STEC or EPEC related genes (isolates 6F8, 9F8, 10F8, 12F8, 24F8 and 30F8). In addition, all six isolates were collected from the same location, and the *stx*1, *stx*2 and *eae* genes were detected. None of the muscle or water samples tested were positive for the STEC or EPEC markers investigated. Isolates from the fish farms were positive for *ehx*A, *lpf*A$_{O113}$ and *saa* virulence genes. Also, strains from the free-living fish presented *ast*A, *ehx*A, *lpf*A$_{O113}$, *saa*, *efa*1 and *paa* genes. Regarding Stx2 toxin variants, the subtypes *stx*2a, *stx*2c and *stx*2d were observed at the same isolate.



Isolate*	*stx*	*eae*	lpfAO113	ehxA	*saa*	*efa*1	*paa*	*astA*	Phylogeny	Serotype†	*stx2*	ST‡	CC§	Resistence
9F8	1, 2		+						B1	O39:H14	a	301	165	AMP, CFL, TET
30F8	2		+					+	B1	ONT:HNT		2607		AMP, CFL
125F5	2		+	+	+				B1	ONT:H18	a,c,d	3884		all‖, except SUT¶
6F8	2		+						B1	O55:HNT	a,d	446	446	AMP, CFL
10F8			+						A	ONT:H36		942		AMP, CFL, TET
24F8	1	+	+	+		+	+		B1	O116:HNT		16	29	AMP, CFL, TET
12F8	2	+	+	+	+				B1	O116:H36	a,d	58	155	none

* F5: fecal sample from fish farm animal; F8: fecal sample from wild animal; 10F8 is the only aEPEC strain and the others, STEC strains.
† NT: nontypeable
‡ ST: Sequence type from MLST
§ CC: Complement from MLST ST
‖ all: AMP-Ampicillin, CFL-Cephalothin, EST-Streptomycin, GEN-gentamicin, CIP-Ciprofloxacin, CLO - Chloramphenicol, TET - Tetracycline, NIT-Nitrofurantoin, NAL - Nalidixic Acid, CRO –Ceftriaxone, CFO - Cefoxitin, KAN - kanamycin, NOR- Norfloxacin, ENO – Enrofloxacin.
¶ SUT-sulfamethoxazole and trimethoprim

Fig. 1 – A dendrogram representing the genetic similarity relationship and virulence indicators in STEC and aEPEC isolates from fish.

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