Samuel L. Yingst,* Magdi D. Saad,* and Stephen A. Felt*

*US Naval Medical Research Unit No. 3, Cairo, Egypt

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Address for correspondence: Samuel L. Yingst, Naval Medical Research Unit No. 3, PSC 452, Box 115, FPO, AE 09835-0001 yingsts@namru3.med.navy.mil

Classifying *Escherichia coli*

To the Editor: Enteropathogenic *Escherichia coli* (EPEC), 1 of the 6 pathotypes of diarrheogenic *E. coli* (DEC), promotes attaching-effacing lesions in eukaryotic cells. These lesions are mediated by intimin, an outer membrane adhesive protein encoded by the *eae* (*E. coli* attaching-effacing) gene (*1*). EPEC is currently subdivided into typical and atypical subgroups. While typical EPEC carry the EPEC adherence factor plasmid (pEAF) that encodes the bundle-forming pilus (BFP) and a complex regulator of various virulence genes (Per) (*1*), atypical EPEC is devoid of pEAF (or does not express a functional BFP) (*1*,*2*). Typical EPEC expresses the localized pattern of adherence (LA), which is characterized by compact bacterial clusters on HeLa and HEp-2 cells (*1*). Conversely, atypical EPEC most often expresses the LA-like pattern (with loose bacterial clusters) or adherence patterns of other DEC pathotypes (*2*).

Enteroaggregative *E. coli* (EAEC), another DEC pathotype, is identified by the characteristic aggregative pattern of adherence (AA) in HeLa/HEp-2 cells; bacteria attach in aggregates to cell surfaces as well as around cells (*1*,*3*). EAEC colonizes the intestinal mucosa, forming a thick biofilm that favors prolonged colonization and induces malnutrition (*1*–*3*). Actually, this pathotype is heterogeneous regarding the presence of putative virulence genes and has recently been subgrouped into typical and atypical EAEC, which carry and lack AggR (a global regulator of EAEC virulence), respectively (*1*,*3*).

We recently conducted a study at the Instituto de Puericultura e Pediatria Martagão Gesteria in Rio de Janeiro, Brazil, on the etiology of diarrhea affecting children of low socioeconomic status (V.B.C. Girão et al., unpub. data). In the study, all *E. coli* isolates were analyzed regarding their adherence patterns in HeLa cells and the presence of specific virulence genes of the DEC pathotypes, according to previously reported methods (*4*,*5*). Among 481 children (<2 years old) with diarrhea who were examined, 16 (3.3%) carried *E. coli* strains that co-expressed LA and AA (LA/AA), a phenotype not found among strains of 99 control children without diarrhea at the same hospital. The LA/AA phenotype was confirmed in individual colonies of each strain as well as in HEp-2 cells. In both cell lineages, prolonged assays (6 hours) showed that a mature biofilm that masked the LA phenotype had developed.

Although LA/AA co-expression in some human *E. coli* has been previously reported by Bouzari et al. (*6*), further information on these isolates is lacking. Moreover, since the expression of LA and AA is used to classify fecal *E. coli* as typical EPEC and EAEC (*1*,*3*), respectively, the classification of such strains within the DEC pathotypes is difficult. To determine their most appropriate classification, we further characterized the 16 LA/AA strains of our collection (Table). Colony hybridization assays used to search for additional *E. coli* virulence genes (*bfpA*, *perA*, E-*hly*, *daaC*, *cdt*, *cnf*, *hly*, *aggR*, *aggC*, *aafC*, *aap*, *shf*, *irp2*, *pet*, *pic*, *astA*, *pap*, *afa*, *sfa*, *efa*, *paa*, *saa*, *enfA*) (*1*,*3*–*5*,*7*) showed that all strains carried *eae*, *bfpA*, and *perA*, and 13 also carried the EAF sequence (a cryptic pEAF marker). Less commonly found genes were *paa*, *shf*, *irp*2, *astA*, and *efa*, and the remaining genes were absent. BFP expression was confirmed in all strains by immunoblot, and positivity in the fluorescent actin staining assay (*8*) demonstrated that they can produce attaching/effacing lesions. PCR analysis of 4 (α, β, γ, and δ) (*9*) of at least 10 recognized intimin subtypes (*1*) showed that subtype δ was the most frequent. Serotyping (*5*) identi-

Table. Genotypic and phenotypic properties of 16 *Escherichia coli* strains that co-express localized and aggregative patterns of adherence*

Strain	Virulence genes	Intimin subtype	Serotype
98180	*eae*, *bfp*A, EAF, *per*A, *paa*	δ	O2:H45
99137	*eae*, *bfp*A, EAF, *per*A, *paa*	δ	O2:H45
21153	*eae*, *bfp*A, EAF, *per*A, *shf*	δ γ†	O55:H51
21187	*eae*, *bfp*A, EAF, *per*A, *shf*	δ γ†	O55:H51
99253	*eae*, *bfp*A, EAF, *per*A, *shf*	δ γ†	O55:HNM
99329	*eae*, *bfp*A, EAF, *per*A, *shf*	δ γ†	O55:HNM
99197	*eae*, *bfp*A, EAF, *per*A	β δ†	O119:H6
98288	*eae*, *bfp*A, EAF, *per*A, *irp2*	β	O119:H6
22622	*eae*, *bfp*A, EAF, *per*A, *irp2*	β	O119:H6
99336	*eae*, *bfp*A, *per*A, *paa*, *astA*, *efa*	α	O142:H6
98351	*eae*, *bfp*A, EAF, *per*A, *astA*	γ	O145:H45
22652	*eae*, *bfp*A, EAF, *per*A, *irp2*	(–)	O178:H33
99245	*eae*, *bfp*A, *per*A, *paa*, *efa*, *ast*A	α	ONT:H6
98025	*eae*, *bfp*A, *per*A, *paa*	α	ONT:H6
98366	*eae*, *bfp*A, EAF, *per*A, *efa*	β	ONT:H7
22150	*eae*, *bfp*A, EAF, *per*A, *astA*	α	ONT:H10

*Isolated from children with diarrhea. EAF, enteropathogenic *E. coli* adherence factor; –, nontypable with the primers tested.
†Intimin types undetermined because amplification products of the expected size were obtained with 2 intimin pairs of primers.

fied at least 10 distinct serotypes among the 16 strains, which demonstrated that they do not make up a single clone. Two serotypes (O119:H6 and O142:H6) are commonly found among typical EPEC (*2*). Certain typical and atypical EPEC serotypes have been associated with distinct intimin subtypes (*9*). Likewise, our LA/AA strains of the same serotype carried the same intimin subtype. Recently, Carvalho et al. (*10*) detected LA/AA expression in 4 of 21 *eae*-positive *E. coli* strains isolated from monkeys with diarrhea. All 4 strains expressed BFP and lacked the EAF sequence; as in our study, 1 belonged to serotype O142:H6 and carried intimin α.

E. coli classification within the DEC pathotypes has epidemiologic and clinical implications for managing diarrheal diseases. However, finding *E. coli* isolates that co-express LA/AA reiterates the difficulty of assigning bacteria to groups on the basis of their adherence phenotype or genotype (particularly when based on mobile genetic elements). Since our analysis with molecular methods showed that these strains carry more characteristics of typical EPEC and lack the AggR regulon, we propose that they be classified as typical EPEC. Typical EPEC are recognized as pathogens, whereas atypical EAEC are not (*3*). In addition, the ability to simultaneously induce attaching/effacing lesions and biofilm production may increase the potential of these strains to cause diarrhea and prolong bacterial residence in the intestines, thus worsening malnutrition in the patient.

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Dennys M. Girão,*† Valéria B.C. Girão,* Kinue Irino,‡ and Tânia A. Tardelli Gomes§

*Universidade Federal do Rio de Janeiro, Rio de Janeiro, Brazil; †Universidade de São Paulo, São Paulo, Brazil; ‡Instituto Adolfo Lutz, São Paulo, Brazil; and §Universidade Federal de São Paulo, São Paulo, Brazil

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Address for correspondence: Tânia A. Tardelli Gomes, Departamento de Microbiologia, Imunologia e Parasitologia, Universidade Federal de São Paulo, Escola Paulista de Medicina Rua Botucatu, 862, 3° andar Vila Clementino, São Paulo, São Paulo, Brazil CEP 04023-062; email: tatgomes@ecb.epm.br

Toscana Virus RNA in *Sergentomyia minuta* Flies

To the Editor: Toscana virus (TOSV) (family *Bunyaviridae*, genus *Phlebovirus*) is an arthropodborne virus transmitted by sandflies. Reports of infections in travelers, clinical research, and epidemiologic studies have shown that TOSV affects the central nervous system and is a major cause of meningitis and encephalitis in Mediterranean countries in which the virus circulates (*1*). In central Italy, this virus is the primary cause of meningitis from May to October, far exceeding enteroviruses as a cause of disease (*2*). In other northern Mediterranean countries, TOSV is among the 3 most prevalent viruses associated with meningitis during the warm seasons (*1*). TOSV has recently been associated with human disease in France (*3*,*4*) and was originally isolated in Italy from *Phlebotomus perniciosus*, then from *P. perfiliewi*, but never from *P. papatasi*. TOSV has also been isolated from the brain of a bat in areas where *P. perniciosus* and *P. perfiliewi* were present, but no hemagglutination-inhibiting antibodies were found in sera from these bats (*5*). In Spain, 2 isolates of TOSV were recovered from 103 pools of sandflies; sequence analyses showed that they were genetically divergent from the Italian strains (*6*). To date, TOSV had not been isolated from sandflies collected in France.

In July 2005, a total of 123 *Sergentomyia minuta* were collected in a 4-day period near Marseille, southeastern France. This work was part of a larger collaborative study, the results of which will be published separately. CDC miniature light traps (John W. Hock Co., Gainesville, FL, USA) were adapted to sandflies with an ultra-fine mesh. Traps were hung 1–2 m above the ground. They were placed in the late afternoon inside or near animal housing facilities (for chickens, rabbits, goats, or horses) in the suburbs of Marseille for 4 successive nights. In these areas, large numbers of geckos were noticed. Each morning, sandflies were collected, identified morphologically, and placed in 1.5-mL Eppendorf tubes. *S. minuta* flies were identified by appearance, and genus was confirmed by sequence analysis, as previously reported (*7*).

Five pools of the captured *S. minuta* were prepared with a maximum of 30 flies per pool. They were ground in 20% fetal bovine serum–enriched phosphate-buffered saline in a Mixer Mill MM300 (Qiagen, Courtaboeuf, France) with one 3-mm tungsten bead and clarified by low-speed centrifugation. We used 200 µL supernatant for total RNA purification onto the MagNAPure platform with the MagNA Pure LC RNA High Performance Kit (Roche Diagnostics, Meylan, France). We used 10 µL RNA suspension for reverse transcription PCR, with primers targeting either a consensus sequence for the phlebovirus polymerase gene (L RNA segment) or Toscana virus (*8*) and the nucleoprotein (N) gene (S RNA segment) specifically (*9*).

Only 1 TOSV-positive pool was observed with primers specific to TOSV polymerase and N genes, respectively. A positive result was observed with primers NPhlebo2+ and ATos2, previously found to target polymerase genes of a range of phleboviruses (*8*). This result was confirmed by sequence analysis (GenBank accession no. DQ195277), which showed 82.8% and 96% identity at the nucleotide and amino acid level, respectively, with a TOSV isolate from Italy (GenBank accession no. X68414). The same pool also tested positive with primers (5′-CGTRGCAGCCACYTCATTAG-3′ and 5′-GTGTCGGCYGCSTTTG-TTCC-3′) designed in this study from the alignment of the 13 sequences of TOSV retrieved from GenBank (accession nos. are shown in the Figure). Comparing the sequence of this 272-bp PCR product with homologous sequences of selected phleboviruses available in the GenBank database showed 97.4%, 87.1%–88.2%, and 78.7% identity at the nucleotide level with TOSV strains isolated in Italy, TOSV isolated in Spain, and sandfly fever Naples virus (Sabin strain), respectively. Phylogenetic analyses of the N gene indicated that this virus clustered with TOSV strains circulating in Italy and Spain but is most closely related to isolates from Italy (Figure). Comparative analysis within the polymerase gene confirmed these data, but distance analysis with sequences of Spanish TOSV was not possible because genetic data were lacking in public databases. The remaining 400-µL volume of sandfly material was used to attempt virus isolation in Vero cells and by intracerebral injection of