Real-Time PCR-Based Mismatch Amplification Mutation Assay for Specific Detection of CS6-Expressing Allelic Variants of Enterotoxigenic *Escherichia coli* and Its Application in Assessing Diarrheal Cases and Asymptomatic Controls

Subrata Sabui,^a Sanjucta Dutta,^a Anusuya Debnath,^a Avishek Ghosh,^a T. Hamabata,^b K. Rajendran,^a T. Ramamurthy,^a James P. Nataro,^c Dipika Sur,^a Myron M. Levine,^d and Nabendu Sekhar Chatterjee^a

National Institute of Cholera and Enteric Diseases, Kolkata, India^a; National Center for Global Health and Medicine, Tokyo, Japan^b; Department of Pediatrics, University of Virginia School of Medicine, Charlottesville, Virginia, USA^c; and Center for Vaccine Development, University of Maryland School of Medicine, Baltimore, Maryland, USA^d

Enterotoxigenic *Escherichia coli* (ETEC) expressing the colonization factor CS6 is widespread in many developing countries, including India. The different allelic variants of CS6, caused by point mutations in its structural genes, *cssA* and *cssB*, are designated AIBI, AIIBII, AIIIBI, AIBII, and AIIIBII. A simple, reliable, and specific mismatch amplification mutation assay based on real-time quantitative PCR (MAMA-qPCR) was developed for the first time for the detection of CS6-expressing ETEC, along with the identification of allelic variations. The assay was based on mismatched nucleotide incorporation at the penultimate base at the 3' ends of the reverse primers specific for *cssA* and *cssB* and was validated using 38 CS6-expressing ETEC isolates. This strategy was effective in detecting all the alleles containing single-nucleotide polymorphisms. Using MAMA-qPCR, we also tested CS6 allelic variants in 145 ETEC isolates from children with acute diarrhea and asymptomatic infections, with the latter serving as controls. We observed that the AIBI and AIIIBI allelic variants were mostly associated with cases rather than controls, whereas the AIIBII variants were detected mostly in controls. In addition, the AIBI and AIIIBI alleles were frequently associated with ETEC harboring the heat-stable toxin gene (*est*) alone or with the heat-labile toxin gene (*elt*), whereas the AIIBII allele was predominant in ETEC isolates harboring the *elt* gene. This study may help in understanding the association of allelic variants in CS6-expressing ETEC with the clinical features of diarrhea, as well as in ETEC vaccine studies.

Enterotoxigenic *Escherichia coli* (ETEC), *Vibrio cholerae* O1, and rotavirus account for the majority of acute diarrhea cases, which can turn fatal in the absence of timely intervention. The virulence factors of ETEC include one or both of the two enterotoxins (6, 14) commonly known as heat labile (LT) and heat stable (ST), depending on their temperature susceptibility, and a group of cell surface proteins collectively called colonization factor antigens (CFAs). The CFAs enable the organisms to initiate pathogenesis by adhering to host intestinal cells and overcoming intestinal peristalsis and the mucus barrier (15, 18). Several studies indicate that 20 to 40% of individuals from developed countries experience ETEC-mediated diarrhea after or while traveling to developing countries where diarrheal pathogens are endemic (4). According to the World Health Organization (WHO), ETEC is the second most common cause of diarrhea, after rotavirus, in children less than 5 years of age, and that is why this age group is the main target for vaccine implementation (8).

CS6 is one of the major CFAs expressed by ETEC. To date, more than 20 CFAs are known, of which CS6 is the most prevalent globally (6, 5, 19). Recent studies indicate that infections caused by CS6-expressing ETEC strains account for up to 20% of the total diarrheal cases in Southeast Asia (1). Due to the increase in the incidence of ETEC-mediated diarrheal disease, considerable attention has been paid to the development of simple, sensitive, and less expensive molecular methods for rapid identification of the pathogen. Currently, CFAs are detected by conventional PCR assay, replacing the conventional immunological tests (7, 12, 13, 16, 17).

The CS6 operon is composed of four genes, *cssA*, *cssB*, *cssC*, and

cssD. The structural genes of CS6 are *cssA* and *cssB*, which exhibit variation in different CS6-expressing ETEC isolates (16, 20). Recently, we documented that *cssA* has three alleles (AI, AII, and AIII) and *cssB* has two alleles (BI and BII). BI- and BII-expressing ETEC strains show differential binding with human intestinal epithelial (Caco-2) cells (16). Following identification of CS6-expressing ETEC by PCR- or antibody-based detection methods, molecular techniques, such as allele-specific PCR and DNA sequencing, are helpful in the detection of CS6 variants. However, these methods are laborious and time-consuming for diagnostic purposes. Currently, there is no simple, specific, and rapid detection method for ETEC expressing CS6 alleles.

In this study, we developed a rapid quantitative PCR (qPCR)-based mismatch amplification mutation assay (MAMA) (2) for the detection of CS6 allelic variants that can be completed within 2 h after DNA extraction. Using this new assay, we screened 145 ETEC isolates for different alleles of both *cssA* and *cssB* and examined the association of these alleles with diarrhea and asymptomatic infections.

Received 10 August 2011 Returned for modification 14 October 2011
Accepted 27 December 2011

Published ahead of print 4 January 2012

Address correspondence to Nabendu Sekhar Chatterjee,
nschatterjee@rediffmail.com.

Copyright © 2012, American Society for Microbiology. All Rights Reserved.
doi:10.1128/JCM.05424-11

FIG 1 Sequence alignment of *cssA* and *cssB* subunit genes of CS6-expressing ETEC. Three alleles of *cssA* and two alleles of *cssB* were aligned using clustalW2 software (<http://www.ebi.ac.uk/Tools/msa/clustalw2>). The asterisks indicate the conserved nucleotides, and the gaps indicate single-nucleotide polymorphisms (SNPs). The primer binding sites are indicated by boldface, and mutated nucleotides of the reverse primer are boxed. Identical regions between CS6 alleles are indicated with dotted lines, and nucleotide positions are shown on the right.

MATERIALS AND METHODS

Bacterial strains and culture conditions. Stool specimens were collected from cases of acute diarrhea in children less than 5 years of age from the Infectious Diseases Hospital and B. C. Roy Memorial Hospital for Children, Kolkata, India. Stool specimens were also collected from age- and sex-matched asymptomatic controls from the community. All the specimens were examined for common enteric pathogens using standardized methods (10). Three lactose-fermenting colonies resembling *E. coli* from MacConkey agar plates were subcultured on normal Luria-Bertani agar (Difco, Sparks, MD) plates and screened for diarrheagenic *E. coli* (DEC) as described previously (11, 15). After confirmation, all the ETEC isolates were serotyped using commercially available O and H antisera (Denka Seiken, Tokyo, Japan). All ETEC isolates were stored at -80°C in Luria-Bertani broth (Difco) with 15% glycerol until further use. A well-characterized ETEC CS6 strain, 4266 (7), and a K-12 *E. coli* strain, DH5 α , were used as PCR positive- and negative-control strains, respectively.

Genomic DNA isolation. ETEC isolates were subcultured from -80°C stock on CFA medium (1% Casamino Acids, 0.15% yeast extract, 0.005% MgSO_4 , 0.005% MnCl_2 , pH 7.4). After 24 h of incubation at 37°C , total genomic DNA was isolated using the NucliSens EasyMag machine (bioMérieux, Marcy l'Etoile, France) according to the manufacturer's

protocol. One ETEC isolate per patient was used in this study, and the presence of CS6 was confirmed by PCR as described previously (16).

Primer design for mismatch qPCR. The MAMA primers to detect the sequence polymorphism among *cssAI*, *cssAII*, *cssAIII*, *cssBI*, and *cssBII* were focused on nucleotide positions 169 and 474 for the *cssA* and *cssB* subunit genes of CS6, respectively (Fig. 1A and B). Two conserved forward primers for the *cssA* and *cssB* genes and five allele-specific polymorphism detection primers, RV-*cssAI*, RV-*cssAII*, RV-*cssAIII*, RV-*cssBI*, and RV-*cssBII*, were designed (Table 1). At the 3' end, these allele-specific primers contained G, C, and A for *cssAI*, *cssAII*, and *cssAIII* and C and A for *cssBI* and *cssBII*, respectively. To increase the specificity of the 3' mismatch activity, nucleotide C was incorporated (rather than T or A) as the penultimate nucleotide of the 5'-3' reverse primers of all CS6 subunit alleles. A conserved forward primer (FO-parC) and a reverse primer (RV-parC) of the housekeeping gene *parC* were used as internal controls.

qPCR conditions. qPCR for the detection of mutations was done in a 20- μl reaction volume containing ~ 60 ng of total genomic DNA, $1\times$ PCR SYBR green Master Mix (Applied Biosystems, Foster City, CA), 0.2 μM forward and MAMA reverse primers, and Milli-Q water to make up the final volume. Thirty cycles of qPCR were carried out as follows on an Applied Biosystems 7500 thermal cycler: stage 1 (95°C for 15 min [enzyme

TABLE 1 Oligonucleotide sequences of primers used in MAMA-qPCR assays

Target gene	Location	GenBank accession no.	Primer	Oligonucleotide sequence ^a	Amplicon size (bp)
<i>cssAI</i>	55 to 189	GQ241334	FO- <i>cssA</i>	5'-AGAACAGAAATAGCGACTA-3'	135
			RV- <i>cssAI</i>	5'-GCTCACACTAAATAATAACC-3'	
<i>cssAII</i>	55 to 189	GQ241330	FO- <i>cssA</i>	5'-AGAACAGAAATAGCGACTA-3'	135
			RV- <i>cssAII</i>	5'-GCTCACACTAAATAATAACG-3'	
<i>cssAIII</i>	55 to 189	GQ241332	FO- <i>cssA</i>	5'-AGAACAGAAATAGCGACTA-3'	135
			RV- <i>cssAIII</i>	5'-GCTCACACTAAATAATAACT-3'	
<i>cssBI</i>	328 to 494	GQ241335	FO- <i>cssB</i>	5'-ATCAGAAAGGATTCTGGC-3'	167
			RV- <i>cssBI</i>	5'-GTAAATGATACACTCAAACG-3'	
<i>cssBII</i>	328 to 494	GQ241337	FO- <i>cssB</i>	5'-ATCAGAAAGGATTCTGGC-3'	167
			RV- <i>cssBII</i>	5'-GTAAATGATACACTCAAAC-3'	
<i>parC</i>	63 to 162	EU561348	FO- <i>parC</i>	5'-TGGATCGTGC GTT GCC GTT TATTG-3'	100
			RV- <i>parC</i>	5'-AATTTGGCGGTAGCATT CAGACCC-3'	

^a Boldface indicates a mismatched nucleotide incorporated in the reverse primer.

activation)], followed by stage 2 (30 cycles of 95°C for 20 s and 49°C for 35 s) and stage 3 (60°C for 45 s) (dissociation). Data for analysis were collected at the last step of stage 2. The melting curve was also analyzed at the dissociation step of stage 3 in each experiment to understand the primer dissociation from the template.

Conventional PCR. ETEC toxin genes that encode LT and ST were detected by multiplex PCR as described previously (11). To check for nonspecific amplicons, a conventional PCR using the same MAMA primers for all the CS6 alleles was done. PCR was performed in a 20- μ l volume consisting of 2 $\times$ PCR Master Mix (Qiagen Fast Cycling PCR Kit; Qiagen, California) and 0.2 μ M forward and MAMA reverse primers. The PCR conditions were as follows: 95°C for 5 min for initial activation of HotStar *Taq* plus DNA polymerase; 28 cycles of 96°C for 5 s, 51°C for 5 s, and 68°C for 10 s; and a final extension step of 1 min at 72°C in an Eppendorf thermal cycler. The amplified products were visualized on a 3% agarose gel after ethidium bromide staining using the Geldoc system (Bio-Rad, Hercules, CA).

Statistical analysis. CS6 allelic variants in cases and controls were tested by univariate analysis and χ^2 exact tests. A probability level (*P*) value of ≤ 0.05 was considered statistically significant.

RESULTS

Specificity of MAMA-qPCR primer pairs. To determine the specificity of MAMA-qPCR, we used the DNA of 38 previously characterized CS6-positive ETEC isolates. CS6-negative ETEC isolates and non-ETEC isolates, such as *V. cholerae*, enteropathogenic *E. coli*, enteroaggregative *E. coli*, and *Campylobacter* spp., were included in the assay. As expected, the assay was negative for CS6-negative ETEC and non-ETEC isolates and was positive only for known CS6-positive ETEC isolates. Allelic variations were detected in all the CS6-positive ETEC isolates. The internal test control, *parC*, gave positive threshold cycle (*C_T*) values in the cases of enteropathogenic *E. coli*, enteroaggregative *E. coli*, and all the ETEC isolates (data not shown). A representative gel picture for CS6 allelic variants is shown in Fig. 2. Our results indicate that the MAMA primers were CS6 allele specific and did not cross-react with other bacterial genes tested in the study.

MAMA-qPCR-based identification of CS6 allelic variants among ETEC isolates. Thirty-eight previously characterized CS6-

FIG 2 Agarose gel of MAMA PCR products for CS6 allelic variants. (A) Lanes 1, 2, 3, 4, and 5 represent alleles AI, AII, AIII, BI, and BII from strain IDH00200, respectively. Lanes 6, 7, 8, 9, and 10 represent alleles AI, AII, AIII, BI, and BII from strain SD173, respectively. Lanes 11, 12, 13, 14, and 15 represent alleles AI, AII, AIII, BI, and BII from strain SD276, respectively. (B) Lanes 1, 2, and 3 represent the *parC* genes from strains IDH00200, SD173, and SD276, respectively.

TABLE 2 ETEC-CS6 allelic variants detected by MAMA-qPCR from cases and controls

CS6 allelic variant	No. of CS6-positive ETEC isolates		OR (95% CI)	P value
	Cases (n = 58)	Controls (n = 13)		
AIBI	36	4	3.68 (0.88–16.41)	0.039 ^a
AIIBI	16	2	2.10 (0.37–15.40)	0.298
AIIBII	2	4	0.08 (0.01–0.63)	0.009 ^b
AIBII	1	2	0.10 (0.00–1.55)	0.084
AIIBII	3	1	0.65 (0.05–17.84)	0.563

^a Statistically significant association with cases.^b Statistically significant association with control.

positive ETEC isolates were selected to validate the MAMA-qPCR assay. In all these isolates, CS6 alleles had been confirmed by DNA sequencing (16). Using MAMA-qPCR, we screened an additional 105 ETEC isolates from diarrheal cases and 40 from controls. Overall, 49% of the ETEC isolates expressed CS6. In the MAMA-qPCR, AIBI (*n* = 40), AIIBI (*n* = 18), AIIBII (*n* = 6), AIBII (*n* = 3), and AIIBII (*n* = 4) were detected in ETEC isolates (Table 2). More of the ETEC isolates from diarrheal cases (34%) than controls (10%) harbored the AIBI allele. The AIBI variant was found with approximately 9 times higher frequency (odds ratio [OR] = 3.68 [95% confidence interval {CI} = 0.88 to 16.41]; *P* = 0.039) among cases than in controls; the frequency of the AIIBI variant was 8 times higher in cases than in controls (OR = 2.10 [95% CI = 0.37 to 15.40]; *P* = 0.298). Conversely, the occurrence of the AIIBII variant was more common in controls (10%) than in cases (2%), which was highly significant (*P* < 0.001). In this study, AIBII and AIIBII variants were detected in very low numbers and the AIBI variant was not found among ETEC isolates.

Correlation between CS6 allelic variants and toxin genes. We also assessed toxin profiles among ETEC isolates by a multiplex PCR method (11) targeting the *elt* and *est* genes and correlated their occurrence with the specific CS6 allele (Table 3). It was found that 28 (48%) of the CS6-expressing ETEC isolates from diarrheal cases harbored *elt* and *est* genes, of which 22 (76%) were the AIBI allelic variant, 5 (17.2%) were AIIBI, and one belonged to the AIIBII variant. Out of 13 ETEC isolates from controls, 3 harbored CS6 as well as *elt* and *est* genes. Among 16 *est*-positive ETEC isolates from diarrheal cases, 6 possessed the AIBI allele, 7 the AIIBI allele, 2 the AIIBII allele, and 1 the AIIBII allele. From controls, only 4 ETEC isolates were positive for the *est* gene. The *elt* gene was detected in 13 ETEC isolates from cases, of which 8 were the AIBI allele, 4 were AIIBI, and one was AIBII. Interestingly, most of the AIIBII variants were found among ETEC isolates from controls that harbored only *elt*. The most commonly detected toxin-encoding-gene profiles among CS6 allelic variants in order of frequency were *elt* + *est* > *est* > *elt*. Most of the AIBI allelic variants among cases harbored both *est* and *elt*. The CS6-negative ETEC isolates mostly harbored *est* (data not shown). The serotyping results showed that the majority of ETEC isolates belonged to diverse serotypes (data not shown).

DISCUSSION

Allelic variation in CS6-expressing ETEC strains is a recent observation that has several implications for ETEC epidemiology (16). The screening of these CS6 allelic variants is critical for under-

TABLE 3 Relationship between toxin genes and CS6 allelic variants of CS6-expressing ETEC isolates

CS6 allelic variant	No. of isolates ^a					
	Case ^b (<i>n</i> = 58)			Control ^c (<i>n</i> = 13)		
	<i>elt</i> + <i>est</i>	<i>elt</i>	<i>est</i>	<i>elt</i> + <i>est</i>	<i>elt</i>	<i>est</i>
AIBI	22	8	6	2	2	–
AIIBI	5	4	7	1	–	1
AIIBII	1	–	1	–	4	–
AIBII	–	1	–	–	–	2
AIIBII	–	–	2	–	–	1

^a *n*, total number of CS6-expressing ETEC isolates. The dashes indicate that ETEC isolates were not detected for that toxin type.^b CS6-expressing ETEC was isolated from diarrheal patients as a mixed or sole pathogen.^c CS6-expressing ETEC was isolated from healthy children as a mixed or sole pathogen.

standing the correlation of microevolutionary changes in the pathogen with the clinical severity of the disease and has implications for ETEC vaccine development. Currently, DNA sequencing is the definitive method for identification of CS6 allelic variants. Sequencing is not practical in studies involving large numbers of samples, and it is desirable to develop a method that is rapid, reliable, and cost-effective in clinical and community settings. Considering the number of existing CS6 allelic variants, real-time PCR can be used as an effective alternative to DNA sequencing. MAMA-PCR has become a powerful tool for scoring of allelic variation. Previously, this technique was used for the differentiation of cholera toxin B subunit in the newly emerged El Tor hybrid strains of *V. cholerae* O1 that have spread in several regions of endemicity (9). In this study, we developed a simple, specific, reliable, and cost-effective MAMA-qPCR assay for the differentiation of CS6-expressing ETEC variants that can detect nanogram levels of DNA. It costs about \$10 per sample, which is much less than DNA sequencing costs. To simplify the assay further, DNA extraction by a simple heating method may also be adopted.

In addition to the formulation of this MAMA-qPCR, we applied the assay to ETEC isolates from children less than 5 years of age with acute diarrhea and age- and sex-matched controls from the urban community. Many studies with ETEC have shown that CS6 is the most prevalent CFA type in developing countries (3, 7, 12, 16). Overall, the AIBI variant was the most prevalent type among CS6-expressing ETEC isolates in Kolkata. This inference should be further confirmed using a greater number of ETEC isolates from diarrheal cases and healthy controls. It was also observed that AIBI was the most frequently detected CS6 variant in ETEC isolates from cases, whereas the AIIBII variant was mainly found in the control group. Simultaneous occurrence of LT and ST toxin types in ETEC was significantly higher among the AIBI variant, whereas the AIIBII variant of ETEC was associated with LT alone. Previous epidemiological studies with ETEC have shown that LT toxin-producing ETEC were found significantly more often among controls than was the ST/ST plus LT toxin type (12). In our previous study, we showed that in the presence of the BII allele, CS6-expressing ETEC binds to Caco-2 cells with less affinity than BI allele-expressing ETEC (16). Based on our findings, it is possible that the LT-ETEC is associated with less severe clinical cases due to the lower adherence ability of this allelic type. Further molecular and epidemiological studies are required to prove this presumption.

In summary, the MAMA-qPCR method is a reliable, cost-effective, and simple method for the detection of CS6-expressing ETEC isolates and their allelic variants. The assay also provides information regarding the severity of ETEC-induced diarrhea and the development of vaccines against CS6-expressing ETEC.

ACKNOWLEDGMENTS

This study was supported in part by grants from the Program of Founding Research Center for Emerging and Reemerging Infectious Diseases, Ministry of Education, Culture, Sports, Science and Technology of Japan, and the Global Enteric Multicenter Study, University of Maryland, Baltimore, MD, and a Grant-in-Aid from the Indian Council of Medical Research (ICMR), India.

We thank Charles Sohaskey, VA Medical Center—Long Beach, Long Beach, CA, for reading the manuscript.

REFERENCES

- Black RE, et al. 2010. Global, regional, and national causes of child mortality in 2008: a systematic analysis. *Lancet* 375:1969–1987.
- Cha RS, Zarbl H, Keohavong P, Thilly WG. 1992. Mismatch amplification mutation assay (MAMA): application to the c-H-ras gene. *PCR Methods Appl.* 2:14–20.
- Cohen D, et al. 2010. Phenotypic characteristics of enterotoxigenic *Escherichia coli* associated with acute diarrhea among Israeli young adults. *Foodborne Pathog. Dis.* 7:1159–1164.
- DuPont HL, et al. 1971. Pathogenesis of *Escherichia coli* diarrhea. *N. Engl. J. Med.* 285:1–9.
- Qadri F, Svennerholm AM, Faruque ASG, Sack RB. 2005. Enterotoxigenic *Escherichia coli* in developing countries: epidemiology, microbiology, clinical features, treatment, and prevention. *Clin. Microbiol. Rev.* 18:465–483.
- Gaastera W, Svennerholm AM. 1996. Colonization factors of human enterotoxigenic *Escherichia coli* (ETEC). *Trends. Microbiol.* 4:444–452.
- Ghosal A, Bhowmick R, Nandy RK, Ramamurthy T, Chatterjee NS. 2007. PCR-based identification of common colonization factor antigens of enterotoxigenic *Escherichia coli*. *J. Clin. Microbiol.* 45:3068–3071.
- Lanata CF, Mendoza W, Black RE. 2002. Improving diarrhoea estimates. WHO, Geneva, Switzerland.
- Morita M, et al. 2008. Development and validation of a mismatch amplification mutation PCR assay to monitor the dissemination of an emerging variant of *Vibrio cholerae* O1 biotype El Tor. *Microbiol. Immunol.* 52:314–317.
- Nair GB, et al. 2010. Emerging trends in the etiology of enteric pathogens as evidenced from an active surveillance of hospitalized diarrhoeal patients in Kolkata, India. *Gut Pathog.* 2:4.
- Nguyen TV, Van LP, Huy LC, Gia KN, Weintraub A. 2005. Detection and characterization of diarrheagenic *Escherichia coli* from young children in Hanoi, Vietnam. *J. Clin. Microbiol.* 43:755–760.
- Rivera FP, et al. 2010. Genotypic and phenotypic characterization of enterotoxigenic *Escherichia coli* strains isolated from Peruvian children. *J. Clin. Microbiol.* 48:3198–3203.
- Rodas C, et al. 2009. Development of multiplex PCR assays for detection of enterotoxigenic *Escherichia coli* colonization factor and toxins. *J. Clin. Microbiol.* 47:1218–1220.
- Sack RB. 1975. Human diarrheal disease caused by enterotoxigenic *Escherichia coli*. *Annu. Rev. Microbiol.* 29:333–353.
- Sack RB. 1980. Enterotoxigenic *Escherichia coli*: identification and characterization. *J. Infect. Dis.* 142:279–286.
- Sabui S, et al. 2010. Allelic variation in colonization factor CS6 of enterotoxigenic *Escherichia coli* isolated from patients with acute diarrhoea and controls. *J. Med. Microbiol.* 59:770–779.
- Vidal MR, et al. 2009. Characterization of the most prevalent colonization factor antigens present in Chilean clinical enterotoxigenic *Escherichia coli* strains using a new multiplex polymerase chain reaction. *Diagn. Microbiol. Infect. Dis.* 65:217–223.
- Wolf MK. 1997. Occurrence, distribution, and associations of O and H serogroups, colonization factor antigens, and toxins of enterotoxigenic *Escherichia coli*. *Clin. Microbiol. Rev.* 10:569–584.
- Wolf MK, et al. 1989. Characterization of CS4 and CS6 antigen components of PCF8775, a putative colonization factor complex from enterotoxigenic *Escherichia coli* E8775. *Infect. Immun.* 57:164–173.
- Wolf MK, et al. 1997. The CS6 colonization factor of human enterotoxigenic *Escherichia coli* contains two heterologous major subunits. *FEMS Microbiol. Lett.* 148:35–42.