ORIGINAL ARTICLE PARASITOLOGICAL

Detection of intestinal protozoa in paediatric patients with gastrointestinal symptoms by multiplex real-time PCR

L. Maas[1], J. W. Dorigo-Zetsma[2], C. J. de Groot[1], S. Bouter[2], F. B. Plötz[1] and B. E. van Ewijk[1]

1) *Department of Paediatrics, Tergooi Hospitals and* b) *Department of Medical Microbiology, Tergooi Hospitals, Hilversum, The Netherlands*

Abstract

The performance of a multiplex real-time PCR for the detection of *Blastocystis*, *Dientamoeba fragilis*, *Giardia lamblia*, *Cryptosporidium* species and *Entamoeba* species in faecal samples was evaluated in an observational prospective study. Paediatric patients (0–18 years) presenting with gastrointestinal symptoms and suspected of having enteroparasitic disease were included. A questionnaire on gastrointestinal symptoms and the chosen treatment was completed at the start of the study and after 6 weeks. Of 163 paediatric patients (mean age, 7.8 years), 114 (70%) had a PCR-positive faecal sample. *D. fragilis* was detected most frequently, in 101 patients, followed by *Blastocystis* in 49. In faecal samples of 47 patients, more than one protozoan was detected, mainly the combination of *D. fragilis* and *Blastocystis*. Reported gastrointestinal symptoms were abdominal pain (78%), nausea (30%), and altered bowel habits (28%). Eighty-nine of the PCR-positive patients were treated with antibiotics. A significant reduction in abdominal pain was observed both in treated and in untreated patients. This study demonstrated that multiplex real-time PCR detects a high percentage of intestinal protozoa in paediatric patients with gastrointestinal symptoms. However, interpretation and determination of the clinical relevance of a positive PCR result in this population are still difficult.

Keywords: *Blastocystis*, *Dientamoeba fragilis*, gastrointestinal symptoms, intestinal protozoa, multiplex real-time PCR, paediatrics, RT-PCR
Original Submission: 17 May 2013; **Revised Submission:** 29 August 2013; **Accepted:** 30 August 2013
Editor: E. Bottieau
Article published online: 17 October 2013
Clin Microbiol Infect 2014; **20:** 545–550
doi: 10.1111/1469-0691.12386

Corresponding author: B. E. van Ewijk, Department of Paediatrics, Tergooi Hospitals, Box 10016, 1201 DA Hilversum, The Netherlands
E-mail: bvanewijk@tergooiziekenhuizen.nl

Introduction

Gastrointestinal symptoms, such as abdominal pain, nausea, acute or chronic diarrhoea, and altered bowel habits, are frequently seen in paediatric patients. Among several other causes, intestinal protozoa may be involved. However, the actual role of protozoal infections in cases with gastrointestinal symptoms, and therefore the relevance of detection of intestinal protozoa, is a subject of discussion [1–3].

In The Netherlands, the routine diagnostic procedure for detection of intestinal protozoa consists of microscopy on two sodium acetate formalin-preserved stool specimens and on one unpreserved specimen in a so-called triple faeces test (TFT) [4]. Although the TFT has shown to be an effective tool for the detection of intestinal parasites [4], it requires considerable effort. The patient has to collect three stool samples on three consecutive days, and the microbiological laboratory has to examine three samples microscopically. The complexity of the TFT procedure might be one of the reasons why only a limited amount of data on the prevalence, clinical characteristics and treatment outcome of parasitic gastrointestinal illness in paediatric patients is available. Real-time PCR has recently been shown to be a sensitive and specific diagnostic alternative for the detection of intestinal protozoa, and some authors recommend its routine use [5–7]. It is less labour-intensive, and has comparable or higher sensitivity with only one stool sample instead of three, making it an attractive alternative to microscopy. However, no clinical data on the implementation of real-time PCR in daily paediatric practice are available in these or other studies.

This prospective, observational and daily practice study was undertaken to identify intestinal protozoa in faeces of paediatric patients with gastrointestinal symptoms by use of a multiplex real-time PCR and to follow up clinical features 6 weeks after inclusion.

Patients and Methods

The study was carried out in the outpatient paediatric department of a general teaching hospital and in the practices of ten collaborating general practitioners (GPs). Patients were included during a 6-month period, from September 2010 to March 2011. The ethical committee of the hospital approved the study.

Study design

Paediatric patients (0–18 years) with any presentation of gastrointestinal symptoms lasting for >2 weeks and/or paediatric patients clinically suspected of having a parasitic gastrointestinal illness by the treating paediatrician or GP were included if their physician decided to perform PCR to detect intestinal parasites in faeces. Paediatric patients diagnosed with other common causes of gastrointestinal symptoms were excluded. This included the suspicion and detection of gastrointestinal bacteria and viruses, chronic gastrointestinal morbidity (such as inflammatory bowel disease or coeliac disease), recent use of antibiotics (in the past 6 weeks), and immunocompromised status.

All paediatric patients and/or their parents completed a questionnaire about the characteristics of the gastrointestinal symptoms. The questionnaire consisted of questions on the presence of abdominal pain, nausea, acute diarrhoea (more than three loose stools a day, present for <14 days), chronic diarrhoea (diarrhoea lasting for >14 days), altered bowel habits (defined as a change in stool pattern other than diarrhoea), weight loss, vomiting, and anal itching. The severity of abdominal pain was scored on a validated paediatric visual analogue scale (VAS), which scores the severity of pain on a scale from 0 to 10 [8].

After completion of the questionnaire, a multiplex real-time PCR was performed for *Blastocystis*, *Dientamoeba fragilis*, *Giardia lamblia*, *Cryptosporidium* species and *Entamoeba* species on a stool sample collected at home (T0). A week after the first visit (T1), the treating physician communicated (by telephone or at a doctor's visit) the PCR results. As there is a lack of evidence concerning both the criteria for starting treatment and the ideal drug regimen, the choice of whether or not to treat (and with which type of antibiotic) in the case of a positive PCR result was left to the treating physician. Details on treatment were registered. Six weeks after the first visit (T6), all treated and untreated paediatric patients and/or their parents filled out the same questionnaire as on T0 in order to enable follow-up of clinical characteristics, the effect of treatment, or the natural course of the symptoms.

Multiplex real-time PCR for intestinal protozoa

For the multiplex real-time PCR *c.* 200 mg of unpreserved faeces was dissolved in 400 μL of lysis buffer (DXL; Qiagen, Hilden, Germany), and, after storage at −20°C overnight, was used for DNA extraction. Prior to automated DNA extraction, phocin herpesvirus (PhHV-1) was added to the faecal sample to serve as an internal control for determining the efficiency of the PCR and detecting inhibition in the sample [9]. Detection of the five protozoa was performed in two separate PCR reactions per DNA sample. In one reaction, a PCR for *G. lamblia* and *D. fragilis*, including PhHV-1, was performed as described previously [10,11]. In a separate assay, *Blastocystis*, *Cryptosporidium* species, and *Entamoeba* species, including PhHV-1, were amplified [12]. The analytical sensitivity and specificity of the PCRs used have been validated at the Leiden University Medical Centre and Tergooi Hospitals (The Netherlands) [10,11], and confirmed after standardized adjustments to the analysis parameters in RotorGene software (Qiagen). Negative extraction and positive DNA controls for each pathogen were included in all PCR runs.

Statistical analysis

Dichotomous and categorical variables were compared by use of the χ^2-test, and continuous data were analysed with non-parametric tests as applicable. All statistical analyses were performed with SPSS version 20 (SPSS, Chicago, IL, USA). A p-value of <0.05 was accepted as statistically significant. Data are expressed as median and range unless stated otherwise.

Results

A total of 171 paediatric patients (61% with gastrointestinal symptoms lasting for >2 weeks and 39% with clinical suspicion of parasitic gastrointestinal illness) participated in the study; eight patients were excluded because they met one of the exclusion criteria. Real-time PCR was positive in 114 of 163 (70%) of the paediatric patients (Table 1). Because of loss to

TABLE 1. PCR results

PCR-positive	PCR-negative
n = 114 (70%) Ten lost to follow-up	*n* = 49 (30%) Five lost to follow-up

TABLE 2. Patient demographics

	PCR-positive (n = 104)	PCR-negative (n = 44)
Age in years (median)	7.5 (0–18)	6 (0–18)
Sex, no. (%)		
Female	52 (50)	25 (57)
Male	52 (50)	19 (43)

follow-up (n = 15), analysis of symptoms and follow-up was performed on data of 104 PCR-positive and 44 PCR-negative patients (total of 148 patients). The characteristics of these patients are shown in Table 2. In the PCR-positive group (52 females and 52 males; median age, 7.5 years), the most commonly reported gastrointestinal complaint was abdominal pain (78%) (with a mean VAS score of 5.7), followed by nausea (30%) and altered bowel habits (28%). Most paediatric patients reported multiple gastrointestinal symptoms. PCR-negative paediatric patients (25 females and 19 males; mean age, 6.0 years) had comparable gastrointestinal symptoms and VAS scores (Table 3).

Intestinal protozoal infections

We identified a single protozoan in 67 (59%) of PCR-positive paediatric patients, two protozoa in 45 (39%), and three protozoa in two (2%) (Fig. 1). *D. fragilis* was detected most frequently, in 89% (101/114) of PCR-positive patients, followed by *Blastocystis* in 43% (49/114). *G. lamblia* was detected in 9% (10/114) of PCR-positive patients. We detected no single infection with *Cryptosporidium* species and no infection with *Entamoeba* species. The most common combination was *D. fragilis* and *Blastocystis* in cases where two protozoa were detected (Fig. 1).

DNA loads

The median cycle threshold (Ct) values in the real-time PCRs for the three most prevalent protozoa varied: for *D. fragilis*, the median Ct value was 26 (range, 20–39), for *Blastocystis* it was 22 (range, 17–35), and for *G. lamblia* it was 30 (range, 23–38). No association between DNA loads of a particular protozoan and gastrointestinal symptoms was found.

Follow-up

Antibiotic treatment was started at T1 in 89 of 104 PCR-positive paediatric patients. In most of these treated patients (93%), *D. fragilis* was detected in the multiplex PCR. Antibiotic treatment consisted of clioquinol 15 mg/kg/day for 10 days in 57 patients (64%) or metronidazole 30 mg/kg/day for 10 days in 25 patients (28%). In seven patients, treatment consisted of paromomycine or was unknown. In the antibiotic-treated group, abdominal pain was significantly reduced in both frequency and severity according to the VAS score, as were all other reported gastrointestinal symptoms, except for altered bowel habits and weight loss (Table 3). In the untreated group (n = 15), only abdominal pain was significantly diminished after 6 weeks. Finally, in the PCR-negative children (n = 44), we observed spontaneous, significant decreases in several gastrointestinal symptoms, including the severity of abdominal pain (VAS score), after 6 weeks (Table 3).

Discussion

Recent studies have shown that PCR is a technically feasible alternative for detecting intestinal protozoa, with numerous practical advantages [6,7,10,12–14]. However, the interpretation and clinical implications of positive real-time PCR results remain a challenge for the treating physician. We therefore performed a prospective, observational study in a Dutch paediatric and GP setting, identifying intestinal protozoa by PCR in paediatric patients with gastrointestinal symptoms. To our knowledge, this is the first study to prospectively evaluate such a multiplex real-time PCR from a clinical perspective in paediatric patients.

The multiplex real-time PCR used in our study was designed to detect five commonly found protozoa. Almost three-quarters

TABLE 3. Gastrointestinal symptoms at T0 and T6

	Treated patients (n = 89)			Untreated patients (n = 15)			PCR-negative (n = 44)		
Gastrointestinal symptoms, no. (%)	T0	T6	p-value	T0	T6		T0	T6	p-value
Abdominal pain	69 (78)	29 (33)	<0.05	11 (73)	4 (27)	<0.05	36 (82)	22 (50)	<0.05
Nausea	27 (30)	9 (10)	<0.05	4 (27)	2 (13)	NS	12 (27)	5 (11)	<0.05
Altered bowel habits	24 (27)	17 (19)	NS	5 (33)	2 (13)	NS	15 (34)	6 (14)	<0.05
Chronic diarrhoea	15 (17)	5 (6)	<0.05	1 (7)	0	NS	11 (25)	4 (9)	<0.05
Weight loss	12 (14)	6 (7)	NS	2 (13)	0	NS	8 (18)	2 (5)	NS
Anal itching	13 (15)	4 (5)	<0.05	3 (20)	1 (7)	NS	5 (11)	1 (2)	NS
Vomiting	12 (14)	1 (1)	<0.05	2 (13)	0	NS	5 (11)	2 (5)	NS
Acute diarrhoea	11 (12)	1 (1)	<0.05	1 (7)	0	NS	4 (9)	0	NS
Mean VAS score	5.9	4.8	<0.05	5.4	4.5	NS	5.3	4.5	<0.05

NS, not significant; VAS, visual analogue scale.

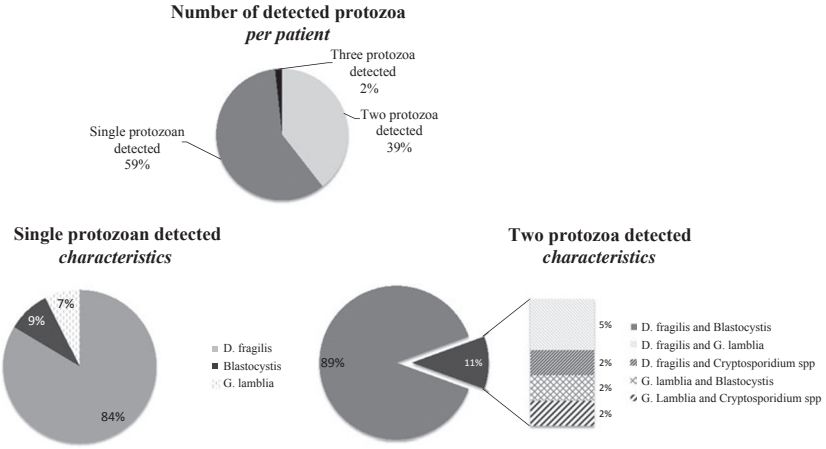

FIG. 1. Distribution of PCR-detected protozoa in 114 paediatric patients with gastrointestinal symptoms.

of the paediatric patients in this study had positive PCR results for one or more intestinal protozoa, with *D. fragilis* being detected in almost all PCR-positive patients. These numbers are significantly higher than those in other studies on intestinal protozoa, especially on dientamoebiasis [2,5,12,15–18]. These differences can be related to study set-up and inclusion criteria, the laboratory techniques used and/or the population studied in cited studies as compared with our study. We also found a high number of cases of simultaneous detection of two or more protozoa. In nearly half of the cases with *D. fragilis*, *Blastocystis* was detected as well. In contrast, *Blastocystis* as a single protozoan was seldom seen. Other studies have shown co-detection in cases of dientamoebiasis, but with significantly lower percentages [5,14]. Gastrointestinal symptoms at enrolment were not statistically significantly different between paediatric patients with positive or negative PCR results. Furthermore, no distinct clinical pattern was related to the presence or the DNA loads of a certain protozoan. These findings are in agreement with previous reports [3,15,19]. In recent decades, the pathogenic potential of *D. fragilis* has been increasingly highlighted [15,18,19], whereas this is less clear in the case of *Blastocystis* [14,20–23], although, for the latter species, discrete subtypes might be related to differences in virulence [21]. Accordingly, most physicians started antiprotozoal treatment cases of a positive PCR result for *D. fragilis*, but not in cases of single detection of *Blastocystis*. After 6 weeks, significant reductions in almost all gastrointestinal symptoms were observed in the PCR-positive and treated patients. However, a significant reduction in abdominal pain was also observed in the PCR-positive, untreated patients and in the PCR-negative patients. As this study was not placebo-controlled or randomized, conclusions on the effect of antibiotic treatment cannot be drawn. However, the spontaneous decrease in symptoms in the untreated PCR-positive patients could possibly be partly explained by single infections with *Blastocystis* and its questionable pathogenic nature. Another explanation could be asymptomatic carriage of *D. fragilis*, as has been described for *G. lamblia* [24,25].

This study has some limitations, mainly owing to the decision to perform an observational study to describe current practice prospectively. As data regarding the detection of intestinal protozoa by PCR and its clinical implications in paediatric patients are lacking, we chose to perform this study as a starting point for formulating further research questions and guiding future study design. The limitations of this study concern the inclusion criteria: the high percentage of PCR-positives that we found in our paediatric study population could be partly the result of selection bias by the treating physician. Furthermore, a healthy control group is lacking; this might, for example, have helped to elucidate the role of asymptomatic carriers. Finally, we did not perform a second PCR at follow-up, as it is not common routine in our clinical practice. Analysis of Ct values from positive PCRs among healthy controls and patient follow-up specimens could also be helpful in defining the diagnostic value of quantitative PCR results. These limitations hamper the drawing of conclusions on the pathogenic role of intestinal protozoa in gastrointestinal symptoms in paediatric patients and the effect of treatment, regarding not only symptomatic relief, but also proven eradication of the protozoa. A randomized placebo-controlled study is needed to further clarify the relationship between the presence of intestinal protozoa and gastrointestinal symptoms in paediatric patients and the effect of treatment.

In conclusion, a multiplex real-time PCR for the detection of intestinal protozoa is a technically feasible tool in a routine microbiological laboratory. In paediatric patients with gastrointestinal symptoms, high percentages of intestinal protozoa were detected, but clinical interpretation and the implications

for treatment will need further research to enable the the development of guidelines for daily clinical practice.

Acknowledgements

We would like to thank E. Luderhoff (GP), who coordinated and facilitated the participation of the GPs. We acknowledge T. E. Matthews for preparing the clinical study, H. Kusters for setting up the molecular diagnostics for intestinal protozoa, and C. Lasham for editing and critically reviewing the manuscript. Preliminary results of this study were presented at the 22nd European Congress of Clinical Microbiology and Infectious Diseases in London, April 2012.

Author Contributions

L. Maas: contributed to the acquisition of the data, drafting of the manuscript, data analysis and interpretation, and critical revision of the article for intellectual content; had full access to all of the data in the study; and approved the final manuscript as submitted. J. Wendelien Dorigo-Zetsma: designed the study; contributed to the acquisition of the data, data analysis and interpretation, drafting of the manuscript, and critical revision of the article for intellectual content; had full access to all of the data in the study; and approved the final manuscript as submitted. C. J. de Groot: designed the study; contributed to the acquisition of the data, and critical revision of the article for intellectual content; and approved the final manuscript as submitted. S. Bouter: contributed to the acquisition of the data; coordinated laboratory diagnostics; and critically reviewed the article for intellectual content, and approved the final manuscript as submitted. F. B. Plötz: contributed to data analysis and interpretation, drafting of the manuscript, and critical revision of the article for intellectual content; and approved the final manuscript as submitted. B. E. van Ewijk: designed the study; contributed to the acquisition of the data, drafting of the manuscript, data analysis and interpretation, and critical revision of the article for intellectual content; had full access to all of the data in the study, and takes responsibility for the integrity of the data and the accuracy of the data analysis; and approved the final manuscript as submitted.

Transparency Declaration

The authors have no conflict of interest related to this manuscript.

References

1. Gijsbers CF, Benninga M, Buller H. Clinical and laboratory findings in 220 children with recurrent abdominal pain. *Acta Paediatr* 2011; 100: 1028–1032.
2. Gijsbers CF, Kneepkens CM, Schweizer JJ, Benninga MA, Buller HA. Recurrent abdominal pain in 200 children: somatic causes and diagnostic criteria. *Acta Paediatr* 2011; 100: e208–e214.
3. Vandenberg O, Peek R, Souayah H *et al.* Clinical and microbiological features of dientamoebiasis in patients suspected of suffering from a parasitic gastrointestinal illness: a comparison of *Dientamoeba fragilis* and *Giardia lamblia* infections. *Int J Infect Dis* 2006; 10: 255–261.
4. Van Gool T, Weijts R, Lommerse E, Mank TG. Triple faeces test: an effective tool for detection of intestinal parasites in routine clinical practice. *Eur J Clin Microbiol Infect Dis* 2003; 22: 284–290.
5. Stensvold CR, Arendrup MC, Molbak K, Nielsen HV. The prevalence of *Dientamoeba fragilis* in patients with suspected enteroparasitic disease in a metropolitan area in Denmark. *Clin Microbiol Infect* 2007; 13: 839–842.
6. Stark D, Al-Qassab SE, Barratt JL *et al.* Evaluation of multiplex tandem real-time PCR for detection of *Cryptosporidium* spp., *Dientamoeba fragilis*, *Entamoeba histolytica*, and *Giardia intestinalis* in clinical stool samples. *J Clin Microbiol* 2011; 49: 257–262.
7. Van Lieshout L, Verweij JJ. Newer diagnostic approaches to intestinal protozoa. *Curr Opin Infect Dis* 2010; 23: 488–493.
8. Buchholz M, Karl HW, Pomietto M, Lynn A. Pain scores in infants: a modified infant pain scale versus visual analogue. *J Pain Symptom Manage* 1998; 15: 117–124.
9. Niesters HG. Clinical virology in real time. *J Clin Virol* 2002; 25(suppl 3): S3–S12.
10. Verweij JJ, Blangé RA, Templeton K *et al.* Simultaneous detection of *Entamoeba histolytica*, *Giardia lamblia*, and *Cryptosporidium parvum* in fecal samples by using multiplex real-time PCR. *J Clin Microbiol* 2004; 42: 1220–1223.
11. Verweij JJ, Mulder B, Poell B, van Middelkoop D, Brienen EAT, van Lieshout L. Real-time PCR for the detection of *Dientamoeba fragilis* in fecal samples. *Mol Cell Probes* 2007; 21: 400–404.
12. Bruijnesteijn van Coppenraet LE, Wallinga JA, Ruijs GJ, Bruins MJ, Verweij JJ. Parasitological diagnosis combining an internally controlled real-time PCR assay for the detection of four protozoa in stool samples with a testing algorithm for microscopy. *Clin Microbiol Infect* 2009; 15: 869–874.
13. Stark D, Beebe N, Marriott D, Ellis J, Harkness J. Detection of *Dientamoeba fragilis* in fresh stool specimens using PCR. *Int J Parasitol* 2005; 35: 57–62.
14. Stensvold CR, Lewis HC, Hammerum AM *et al. Blastocystis*: unravelling potential risk factors and clinical significance of a common but neglected parasite. *Epidemiol Infect* 2009; 137: 1655–1663.
15. Stark D, Barratt J, Roberts T, Marriott D, Harkness J, Ellis J. A review of the clinical presentation of dientamoebiasis. *Am J Trop Med Hyg* 2010; 82: 614–619.
16. Ten Hove R, Schuurman T, Kooistra M, Möller L, van Lieshout L, Verweij JJ. Detection of diarrhoea-causing protozoa in general practice patients in The Netherlands by multiplex real-time PCR. *Clin Microbiol Infect* 2007; 13: 1001–1007.
17. Banik GR, Barratt JL, Marriott D, Harkness J, Ellis JT, Stark D. A case-controlled study of *Dientamoeba fragilis* infections in children. *Parasitology* 2011; 138: 819–823.
18. Barratt JL, Harkness J, Marriott D, Ellis JT, Stark D. A review of *Dientamoeba fragilis* carriage in humans: several reasons why this organism should be considered in the diagnosis of gastrointestinal illness. *Gut Microbes* 2011; 2: 3–12.

19. Johnson EH, Windsor JJ, Clark CG. Emerging from obscurity: biological, clinical, and diagnostic aspects of *Dientamoeba fragilis*. *Clin Microbiol Rev* 2004; 17: 553–570, table of contents.
20. Andiran N, Acikgoz ZC, Turkay S, Andiran F. *Blastocystis hominis*—an emerging and imitating cause of acute abdomen in children. *J Pediatr Surg* 2006; 41: 1489–1491.
21. Stensvold CR, Nielsen HV, Molbak K, Smith HV. Pursuing the clinical significance of *Blastocystis*—diagnostic limitations. *Trends Parasitol* 2009; 25: 23–29.
22. Van Hellemond JJ, Molhoek N, Koelewijn R, Wismans PJ, van Genderen PJ. Is paromomycin the drug of choice for eradication of *Blastocystis* in adults? *J Infect Chemother* 2013; 19: 545–548.
23. Heyland K, Friedt M, Buehr P, Braegger CP. No advantage for antibiotic treatment over placebo in *Blastocystis hominis*-positive children with recurrent abdominal pain. *J Pediatr Gastroenterol Nutr* 2012; 54: 677–679.
24. Al-Mohammed HI. Genotypes of *Giardia intestinalis* clinical isolates of gastrointestinal symptomatic and asymptomatic Saudi children. *Parasitol Res* 2011; 108: 1375–1381.
25. Ish-Horowicz M, Korman SH, Shapiro M *et al.* Asymptomatic giardiasis in children. *Pediatr Infect Dis J* 1989; 8: 773–779.