

Journal of the Hellenic Veterinary Medical Society

Vol 62, No 3 (2011)



Molecular screening for *Blastocystis* sp. in canine faecal samples in Greece

G. SPANAKOS (Γ. ΣΠΑΝΑΚΟΣ), E. PAPADOGIANNAKIS (Ε. ΠΑΠΑΔΟΓΙΑΝΝΑΚΗΣ), V. KONTOS (Β. ΚΟΝΤΟΣ), P. MENOUNOS (Π. ΜΕΝΟΥΝΟΣ), E. VELONAKIS (Ε. ΒΕΛΟΝΑΚΗΣ), C. KOUTIS (Χ. ΚΟΥΤΗΣ), N. C. VAKALIS (Ν. ΒΑΚΑΛΗΣ)

doi: [10.12681/jhvms.14852](https://doi.org/10.12681/jhvms.14852)

To cite this article:

SPANAKOS (Γ. ΣΠΑΝΑΚΟΣ) G., PAPADOGIANNAKIS (Ε. ΠΑΠΑΔΟΓΙΑΝΝΑΚΗΣ) E., KONTOS (Β. ΚΟΝΤΟΣ) V., MENOUNOS (Π. ΜΕΝΟΥΝΟΣ) P., VELONAKIS (Ε. ΒΕΛΟΝΑΚΗΣ) E., KOUTIS (Χ. ΚΟΥΤΗΣ) C., & VAKALIS (Ν. ΒΑΚΑΛΗΣ) N. C. (2017). Molecular screening for *Blastocystis* sp. in canine faecal samples in Greece. *Journal of the Hellenic Veterinary Medical Society*, 62(3), 216–220. <https://doi.org/10.12681/jhvms.14852>

Molecular screening for *Blastocystis* sp. in canine faecal samples in Greece.

Spanakos G.¹ PhD, Papadogiannakis E.^{2*} DVM, CertSAD, PhD, Kontos V.² DVM, PhD, Menounos P.¹ PhD, Velonakis E.³ MD, PhD, Koutis C.⁴ MD, PhD, Vakalis N. C.¹ MD, PhD

¹ Department of Parasitology, Entomology and Tropical Diseases, National School of Public Health, Athens, Greece

² Department of Veterinary Public Health, National School of Public Health, Athens, Greece

³ Department of Microbiology and Applied Immunology, National School of Public Health, Athens, Greece

⁴ Department of Public Health, Technological Education Institute of Athens, Athens, Greece

Μοριακή αναζήτηση της *Blastocystis* sp. σε κόπρανα σκύλου στην Ελλάδα.

Γ. Σπανάκος¹ PhD, Εμμ. Παπαδογιαννάκης^{2*} DVM, CertSAD, PhD, Β. Κοντός² DVM, PhD, Π. Μενούνος³ PhD, Ε. Βελονάκης⁴ MD, PhD, Χ. Κούτης⁵ MD, PhD, Ν. Βακάλης¹ MD, PhD

¹ Τομέας Παρασιτολογίας, Εντομολογίας και Τροπικών Νοσημάτων, Εθνική Σχολή Δημόσιας Υγείας, Αθήνα

² Τομέας Κτηνιατρικής Δημόσιας Υγείας, Εθνική Σχολή Δημόσιας Υγείας, Αθήνα

³ Τομέας Μικροβιολογίας και Εφαρμοσμένης Ανοσολογίας, Εθνική Σχολή Δημόσιας Υγείας, Αθήνα

⁴ Τομέας Δημόσιας Υγιεινής, Τ.Ε.Ι Αθήνας, Αθήνα

ABSTRACT. *Blastocystis* is an anaerobic parasitic microorganism which has been found in the intestinal tract of many vertebrates including humans and dogs. Recently, members of *Blastocystis* sp. were classified into nine sub-types, based on phylogenetic trees derived from sequence analysis of the small sub-unit ribosomal RNA gene. The pathogenesis of *Blastocystis* in the dog remains uncertain and the existence of pathogenic and non-pathogenic sub-types is under investigation. Faecal samples from 30 privately owned and 42 stray dogs, originated from central and northern Greece, were examined by both light microscopy and PCR assays for the detection of *Blastocystis* sp. All samples were found to be negative by both assays. It seems that, in Greece, the dog is unlikely to be a "reservoir" of *Blastocystis* sp., although a large scale epidemiologic study is needed to be carried out in order for more conclusive results to be drawn.

Keywords: *Blastocystis*, dog, light microscopy, PCR, Greece

ΠΕΡΙΛΗΨΗ. Η *Blastocystis* είναι ένα αναερόβιο παράσιτο που έχει βρεθεί στο έντερο πολλών σπονδυλωτών, περιλαμβανομένων του ανθρώπου και του σκύλου. Πρόσφατα, η *Blastocystis* ταξινομήθηκε σε εννέα υπότυπους, βάσει των φυλογενετικών δένδρων που προέκυψαν από την ανάλυση της αλληλουχίας της μικρής υποομάδας του rRNA γονιδίου. Η παθογένεια της *Blastocystis* στο σκύλο παραμένει ασαφής και η ύπαρξη παθογόνων και μη-παθογόνων υπότυπων ακόμη ερευνάται. Δείγματα κοπράνων από 30 δεοποζόμενους και 42 αδέσποτους σκύλους, προερχόμενους από την Κεντρική και Βόρεια Ελλάδα εξετάστηκαν μικροσκοπικά και με τη μέθοδο PCR για τυχόν ανίχνευση της *Blastocystis* sp. Όλα τα δείγματα βρέθηκαν αρνητικά και με τις δύο μεθόδους. Φαίνεται ότι στην Ελλάδα ο σκύλος μάλλον δεν συνιστά «δεξαμενή» της *Blastocystis* sp., αν και απαιτείται επιδημιολογική μελέτη μεγάλης κλίμακας για να εξαχθούν ασφαλή συμπεράσματα.

Λέξεις ευρετηρίασης: *Blastocystis*, σκύλος, μικροσκοπική εξέταση, PCR, Ελλάδα

Correspondence: * Papadogiannakis E.
Department of Veterinary Public Health, National School of Public Health
196 Alexandras Ave, 115 21 Athens, Greece
Tel.: +30 2132010217, Fax: +30 2109818444, E-mail: dermpap1@otenet.gr

Αλληλογραφία: * Ε. Παπαδογιαννάκης
Τομέας Κτηνιατρικής Δημόσιας Υγείας, Εθνική Σχολή Δημόσιας Υγείας
Λεωφ. Αλεξάνδρας 196, 115 21 Αθήνα
Τηλ.: +30 2132010217, Fax: +30 2109818444, E-mail: dermpap1@otenet.gr

Submission date: 14.07.2011
Approval date: 29.07.2011

Ημερομηνία υποβολής: 14.07.2011
Ημερομηνία εγκρίσεως: 29.07.2011

INTRODUCTION

Blastocystis sp. is an anaerobic, single-celled, enteric parasite that inhabits the intestinal tract of both humans and many species of animals. The protozoan was first observed in human faecal samples and was reported as yeast in 1912 (Brumpt 1912). Recently, *Blastocystis* was demonstrated as a member of Stramenopiles (Silberman et al. 1996). It is considered the most common protozoan reported in human faecal samples with surveys revealing infection rates of up to 50% in developing countries, while the prevalence of the infection is lower in developed countries (Windsor et al. 2002, Stenzel and Boreham 1996). *Blastocystis* has, also, been isolated from a wide range of animals, including mammals, birds, amphibians, reptiles and insects. Generally, isolates from humans have been characterized as *B. hominis*, whereas isolates from other species have usually been named *Blastocystis* sp., although a small number of parasites have been named after the host (Belova 1991, Belova 1990, Pakandl 1991, Quilez et al. 1995). Nevertheless, all isolates reported so far, irrespective of their host origin and genetic diversity, are morphologically indistinguishable (Kaneda et al. 2001, Ho et al. 2001, Clark 1997, Yoshikawa et al. 1996, Yoshikawa et al. 1998, Yoshikawa et al. 2000).

The pathogenesis of the organism remains uncertain both in humans and dog. Various clinical reports in humans consider *Blastocystis* as the causative agent of intestinal disease, including diarrhea, inflammatory bowel disease, irritable bowel syndrome and ulcerative colitis, yet the pathogenicity of the parasite has not been equivocally proven (Albrecht et al. 1995, Carrascosa et al. 1996, Levy et al. 1996, Sun et al. 1989, Zaki et al. 1991, Yakoob et al. 2004). There is no evidence so far that *Blastocystis* is pathogenic for dogs, although in one case with intestinal symptoms *Blastocystis* was the only isolate (Chapman et al. 2009). Detection of *Blastocystis* is based on microscopic identification of the parasite in faecal samples, culture, and PCR (Termmathurapoj et al. 2004, Menounos et al. 2008).

Molecular analysis has revealed high polymorphism among *Blastocystis* isolates. Furthermore, isolates with identical genotype have been isolated from humans and animals, indicating cross species contamination. Recently, a consensus on *Blastocystis* terminology was proposed (Stensvold et al. 2007).

According to this consensus, all mammalian and avian *Blastocystis* isolates are designated *Blastocystis* sp. and separated into ten sub-types, named 1-10 according to phylogenetic trees constructed from SSU rRNA sequences.

The prevalence of *Blastocystis* in animal hosts varies among species and geographic location (Duda et al. 1998, Parkar et al. 2007). There are no published data for *Blastocystis* in dogs in Greece, although some anecdotal studies failed to isolate *Blastocystis* from either dogs or cats by light microscopy and culture (Haralabidis 2003). Recently, a PCR method has been found to be highly sensitive and specific for the detection of *Blastocystis* in faecal samples (Menounos et al. 2008).

This study was a preliminary attempt to the detection of *Blastocystis* sp. in faecal samples from both privately owned and stray healthy dogs in Greece.

MATERIALS AND METHODS

Samples

A total of 72 canine faecal samples, randomly selected, were processed. Thirty (30) samples were obtained from privately owned dogs and 42 samples from stray animals of both sexes and of various age and breeds. All samples were collected directly from the rectum between September and October 2008 in 15 ml sterilized plastic tubes. Microscopical examination was accomplished within 6 hours of collection, while PCR was carried out later with the faecal samples being kept in -40°C until processed. All dogs originated from various areas of central and northern Greece.

Light microscopy

All faecal samples were initially examined by light microscopy using faecal suspensions. Approximately, 0.1 g of faecal material was suspended in 1 ml of normal saline and then filtered through gauze. Then, 20 µl of faecal suspension was directly examined under a light microscope at 200x magnification.

DNA isolation

The QIAamp Stool Mini Kit (Qiagen) was used for DNA isolation from faeces, according to the manufacturer's instructions. DNA was eluted with 100 microliters of AE Buffer to increase DNA concentration.

PCR

The PCR was performed as previously described

(Menounos et al. 2008) using the primer pair BLF: 5'-CGAATGGCTCATTATATCAGTT-3' and BLR: 5'-TCTTCGTTACCCGTTACTGC-3'. Briefly, in a final volume of 50 microliters containing 1X PCR buffer, 1.5 mM magnesium chloride, 0.2 μ M of each dNTP (New England Biolabs) and 100 pmoles of each primer (synthesized by Syntezza), 10 microliters of template DNA were added. Cycling was performed in Eppendorf or M-J Research thermocyclers. Cycling conditions included an initial denaturation at 94°C for 5 min, 40 cycles at 94°C for 1 min, 53°C for 1 min and 72°C for 1 min and a final elongation step at 72°C for 10 min. A positive and a negative control were included for each reaction. DNA extracted from microscopically-confirmed positive human faecal sample served as a positive control, while DNA extracted from PCR-confirmed negative human sample served as the negative. PCR products were separated with 2% agarose gel electrophoresis, visualized under UV light and photographed with a digital camera. Standard precautions were applied to avoid PCR contamination. PCR reagents were aliquoted and aerosol barrier tips, dedicated pipette sets, laminar flow hoods and separate laboratory areas were used for each step of the procedure.

SSCP

Fifteen microlitres of PCR products were denatured and electrophorized in polyacrylamide gel (7.2% acrylamide, 0.22% bis, 1.25 \times TBE, 0.1% AP and 0.8 \times 10⁻³ % TEMED) for 25 h at 14°C and 420 V. The bands were visualized with silver stain.

Sequencing

PCR product was electrophorised for approximately 30 min in a 3% agarose gel and the ~250 bp band was excised. DNA was isolated from the gel slice using the QIAquick Gel Extraction Kit (Qiagen) according to the manufacturer's recommendations. DNA was eluted in 30 microliters of EB buffer. Cycle sequencing was performed in a GenAmp System 2700 thermocycler using the ABI Prism BigDye Terminator v3.1 Cycle Sequencing kit (Applied Biosystems) and PCR primers.

RESULTS

Blastocystis cysts could not be detected in any of all samples examined by light microscopy. From the 72 samples examined by PCR, 8 presented a faint band

located between 200 and 300 bp. These PCR products were examined by SSCP and presented an unknown (in relation to those obtained from human samples) pattern. Sequencing of the PCR product resulted in a sequence which presents over 95% homology to *Absidia* (Mycocladius) sequences (GenBank Accession numbers EU826361, GQ169404, GQ169392, AF157117, EU826360, FJ176934, AF113407, AF113408, DQ340176) as determined by Blast (Zheng et al. 2000). The sequence has been sent by our research team and deposited in GenBank with Acc. No HM770856.

DISCUSSION

Applying the previously reported PCR in dog feces, 270 bp bands were produced that could interfere with correct detection of *Blastocystis*. In humans, faecal samples producing a non-*Blastocystis* ~250 bp band have not been found. However, it has already been reported that a ~200 bp band is produced in human faecal samples presenting a high fungal load, thus indicating the possibility of primer hybridization to fungi DNA. In humans, *Absidia* usually does not inhabit the intestine of normal immunocompetent individuals, being mainly isolated from immunocompromised hosts (Ribes et al. 2000). This probably explains why this fungus does not interfere with *Blastocystis* detection in human faecal material.

Although culture was not carried out in our study, it has been shown that the PCR method applied is at least equally sensitive with culture for the detection of *Blastocystis* organisms in faecal samples (Menounos et al. 2008). It has been shown that *Blastocystis* from non-human sources comprise isolates that appear to fall outside the genetic range of the already known 10 subtypes of the parasite (Yoshikawa et al. 2007, Stensvold et al. 2007), although they are closely related. Consequently it is possible that DNA from canine-specific *Blastocystis* strains is not amplified by the currently used PCR method, although this is not supported by microscopical examination. Accordingly, based on the results of this study, it seems that *Blastocystis* is not probably a habitat of the normal canine intestine of dogs in Greece.

Although a small number of publications refer to *Blastocystis* in relation to dogs, there are several contradictory reports. Our findings are in agreement with a similar study conducted in Japan (Abe et al. 2002) and Bangkok (Inpankaew et al. 2007). It should be noted

that only the studies indicating exclusively the absence of *Blastocystis* are included, as various parasitological studies on dog faeces do not refer *Blastocystis* as a finding, but it is not clear if the parasite was ignored, or it was not detected. (Claerebout et al. 2009).

In contrast, presence of *Blastocystis* in dog faecal samples was reported in Australia (Duda et al. 1998), Chile (Javier López et al. 2006) and Iran (Daryani et al. 2008).

It has not been elucidated yet why the parasite prevalence varies considerably from one region to another, but explanations could be the sanitary conditions in which the animals live, the different methodology used and unknown environmental factors. Dogs are known to be infected with *Blastocystis* ST1 and ST3 (Parkar et al. 2007, Stensvold et al. 2009). It should be noted that ST3 is the most frequent subtype found in humans.

Although there have been no published data regarding the prevalence of *Blastocystis* in humans in

Greece, the isolation of the parasite from faecal material, mainly (but not exclusively) from patients with diarrhea, is not unusual in our laboratory. Based on the results of this study, the zoonotic transmission of *Blastocystis* from dogs to humans seems to be unlikely in Greece, although a large scale epidemiological study is needed to confirm this preliminary assumption. Several studies suggest that *Blastocystis* is not host-specific and may be cross-infective among various animal hosts (Yoshikawa et al. 1996, Arisue et al. 2003, Tan 2004), although the extent and nature of zoonotic transmission of the parasite remains unclear as the published evidence is equivocal (Stensvold et al. 2009). According to the current assumption, genetically identical *Blastocystis* parasites should be found in both humans and animals, in order to be considered zoonotic. Further research is needed to elucidate this issue.

Acknowledgements: The authors would like to thank Mrs. Anastasia Bimpa for excellent technical assistance. ■

REFERENCES

- Abe N, Nagoshi M, Takami K, Sawano Y, Yoshikawa H. (2002). A survey of *Blastocystis* sp. in livestock, pets and zoo animals in Japan. *Veterinary Parasitology*, 106: 203-212.
- Albrecht H, Stellbrink HJ, Koperski K, Greden H. (1995). *Blastocystis hominis* in human immunodeficiency virus-related diarrhea. *Scand J Gastroenterol*, 30: 909-914.
- Arisue N, Hashimoto T, Yoshikawa H. (2003). Sequence heterogeneity of the small subunit ribosomal RNA genes among *Blastocystis* isolates. *Parasitology*, 126: 1-9.
- Belova LM, Kostenko LA. (1990). *Blastocystis galli* sp. (Protista: Phizopoda) from the intestine of domestic hens. *Parasitologia*, 24: 164-168.
- Belova LM. (1991). *Blastocystis anatis* sp. nov. (Rhizopoda, Lobosea) from *Anas platyrhynchos*. *Zool Zh*, 70: 5-10.
- Brumpt E. (1912). *Blastocystis hominis* n. sp. et formes voisines. *Bull Soc Pathol Exot*, 5: 725-730.
- Carrascosa M, Martinez J, Perez-Castrillon JL. (1996). Hemorrhagic proctosigmoiditis and *Blastocystis hominis* infection. *Ann Intern Med*, 124: 278-279.
- Chapman S, Thompson C, Wilcox A, and Russell K. (2009). What is your diagnosis? Rectal scraping from a dog with diarrhea. *Vet Clin Pathol*, 38(1): 59-62.
- Claerebout E, Casaert S, Dalemans AC, De Wilde N, Levecke B, Vercruysse J, Geurden T (2009). *Giardia* and other intestinal parasites in different dog populations in Northern Belgium. *Vet Parasitol*, 161(1-2): 41-6.
- Clark CG. (1997). Extensive genetic diversity in *Blastocystis hominis*. *Mol Biochem Parasitol*, 87: 79-83.
- Daryani A, Sharif M, Amouei A, Ettehad GH, Ziaei H, Goharidehi SH, Bastani R (2008). *Blastocystis* spp: a Neglected Zoonotic Protozoan. *Proc ASEAN Congr Trop Med Parasitol*, 3: 59-62.
- Duda A, Stenzel D, Boreham PF. (1998). Detection of *Blastocystis* sp. in domestic dogs and cats. *Veterinary Parasitology*, 76: 9-17.
- Ho LC, Jeyaseelan K, Singh M. (2001). Use of the elongation factor-1 α gene in a polymerase chain reaction-based restriction-fragment-length polymorphism analysis of genetic heterogeneity among *Blastocystis* species. *Mol Biochem Parasitol*, 112: 287-291.
- Inpankaew T, Traub R, Thompson R.C and Sukthana Y (2007). Canine Parasitic Zoonoses in Bangkok Temples. *Southeast Asian J Trop Med Public Health*, 38 (2): 247-255.
- Javier López D, Katia Abarca V, Patricio Paredes M, Elisa Inzunza T (2006). Intestinal parasites in dogs and cats with gastrointestinal symptoms in Santiago, Chile. *Rev Méd Chile*, 134: 193-200.
- Kaneda Y, Horiki N, Cheng XJ, Fujits Y, Msruyama M, Tachibana H. (2001). Ribodemes of *Blastocystis hominis* isolated in Japan. *Am J Trop Med & Hygiene*, 65: 393-396.
- Levy Y, George J, Shoenfeld Y. (1996). Severe *Blastocystis hominis* in an elderly man. *J Infect*, 33: 57-59.
- Menounos PG, Spanakos G, Tegos N, Vassalos CM, Papadopoulou C and Vakalis NC. (2008). Direct detection of *Blastocystis* sp. in human faecal samples and subtype assignment using single strand conformational polymorphism and sequencing. *Mol Cell Probes*, 22: 24-29.
- Pakandl M. (1991). Occurrence of *Blastocystis* sp. in pigs. *Folia Parasitol*, 38: 297-301.
- Parkar U, Traub RJ, Kumar S, Mungthin M, Vitali S, Leelayoova S, Morris K, Thompson RCA. (2007). Direct characterization of *Blastocystis* from faeces by PCR and evidence of zoonotic potential.

- Parasitology, 134(3): 359-67.
- Quilez J, Sanchez-Acedo C, Clavel A, Causape AC. (1995). Occurrence of *Blastocystis* sp. in cattle in Aragon, northeastern Spain. *Parasitol Res*, 81: 703-705.
- Ribes JA, Vanover-Sams CL, Baker DJ. (2000). Zygomycetes in human disease. *Clin. Microbiol. Rev*, 13: 236-301.
- Silberman JD, Sogin ML, Leipe DD, Clark CG. (1996). Human parasite finds taxonomic home. *Nature*, 380: 398.
- Stensvold CR, Suresh GK, Tan KSW, Thompson RCA, Traub RJ, Viscogliosi E, Yoshikawa H, Clark CG. (2007). Terminology for *Blastocystis* sub-types – a consensus. *Trends Parasitol*, 23: 93-96.
- Stensvold CR, Alfellani MA, Norkov-Lauritsen S, Prip K, Victory EL, Maddox C, Nielsen HV, Clark CG. (2009). Sub-type distribution of *Blastocystis* isolates from synanthropic and zoo animals and identification of a new sub-type. *International Journal of Parasitology*, 39: 473-479.
- Stenzel DJ, Boreham PFL. (1996). *Blastocystis hominis* revised. *Clin Microbiol Rev*, 9: 563-584.
- Sun T, Katz S, Tanenbaum B, Schenone C. (1989). Questionable clinical significance of *Blastocystis hominis* infection. *Am J Gastroenterol*, 84: 1543-1547.
- Tan KSW. (2004). *Blastocystis* in humans and animals: new insights using modern methodologies. *Vet. Parasitol*, 126: 121-144.
- Termmathurapoj S, Leelayoova S, Aimpun P, Thathaisong U, Nimmanon T, Taamasri P, Mungthin M. (2004). The usefulness of short-term in vitro cultivation for the detection and molecular study of *Blastocystis hominis* in stool specimens. *Parasitol Res*, 93: 445-447.
- Windsor JJ, MacFarlane L, Hughes-Thapa G, Jones KA, Whitside TM. (2002). Incidence of *Blastocystis hominis* in faecal samples submitted for routine microbiological analysis. *Br J Biomed Sci*, 59: 154-157.
- Yakoob J, Jafri W, Jafri N, Khan R, Islam M, Beg MA, Zaman V. (2004). Irritable bowel syndrome: in search of an etiology: role of *Blastocystis hominis*. *Am J Trop Med Hyg*, 70: 383-385.
- Yoshikawa H, Nagano L, Singh M, Takahashi Y. (1996). DNA polymorphism revealed by arbitrary primers polymerase chain reaction among *Blastocystis* strains isolated from humans, a chicken and a reptile. *J Eukaryot Microbiol*, 3: 127-130.
- Yoshikawa H, Nagano L, Wu Z, Yap EH, Singh M, Takahashi Y. (1998). Genomic polymorphism among *Blastocystis hominis* strains and development of sub-type-specific diagnostic primers. *Mol Cell Probes*, 12: 153-159.
- Yoshikawa H, Abe N, Iwasaka M, Kitano S, Nagano L, Wu Z, Takahashi Y. (2000). Genomic analysis of *Blastocystis hominis* strains isolated from two long-term health care facilities. *J Clin Microbiol*, 38: 1324-1330.
- Yoshikawa H, Wu Z, Hashimoto T, Geok-Choo N, Tan KS. (2007). Ultrastructural and phylogenetic studies on *Blastocystis* isolates from cockroaches. *J. Eukaryot. Microbiol*, 54: 33-37.
- Zaki M, Daud A.S, Pugh R.N.H, Al-Ali F, Al-Mutairi G, Al-Saleh Q. (1991). Clinical report of *Blastocystis hominis* infection in children. *J Trop Med Hyg*, 94: 118-122.
- Zheng Zhang, Scott Schwartz, Lukas Wagner and Webb Miller (2000). "A greedy algorithm for aligning DNA sequences". *J Comput Biol*, 7(1-2): 203-14.
- Χαραλαμπίδης ΣΘ. (2003). Παρασιτικά Νοσήματα ζώων και ανθρώπου. Πρωτοζωώσεις, Ελμινθώσεις, Αρθροποδώσεις. University Studio Press, Θεσσαλονίκη pp: 239-250.

