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PARK2/PACRG polymorphisms and susceptibility to typhoid and paratyphoid fever

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Summary

Host genetic factors may contribute to susceptibility to and outcome in infectious diseases. Recently polymorphisms in *PARK2/PACRG*, a gene cluster linked to ubiquitination and proteasome-mediated protein degradation, were found to be associated with manifest infection by *M. leprae*. Here, we address whether these polymorphisms are associated with susceptibility to infection with *Salmonella typhi* and *S. paratyphi A*, intracellular pathogens that upon infection of humans share with mycobacteria aspects of the hosts' immune response. The polymorphisms of *PARK_e01(-697)*, *PARK_e01(-2599)*, *rs1333955* and *rs1040079* were analysed by polymerase chain reaction and restriction fragment length polymorphism in a case-control study of typhoid and paratyphoid fever patients in an endemic area in Jakarta, Indonesia. For this study, samples were obtained from patients with blood culture-confirmed typhoid fever ($n = 90$), paratyphoid fever ($n = 26$) and fever controls ($n = 337$) in a passive, community-based surveillance and compared to those of randomly selected community controls ($n = 322$) from the same city area. The *PARK_e01(-2599)* allele T was significantly associated with typhoid and paratyphoid fever (OR: 1.51, 95%CI: 1.02–2.23) but the other polymorphisms, *PARK_e01(-697)*, *rs1333955* and *rs1040079*, were not associated. Although within the *PARK2/PACRG* gene cluster the *PARK_e01(-2599)* allele T was most strongly associated with leprosy (OR= 3–5), the association with typhoid is much less strong. Our findings suggest that this polymorphism in *PARK2/PACRG* plays a small but significant role in susceptibility to the intracellular pathogens *S. typhi* and *S. paratyphi*.

Keywords: *PARK2*, *PACRG*, *S. typhi*, *S. paratyphi*, typhoid fever, gene polymorphism

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Introduction

Typhoid fever constitutes a serious public health problem in the world, especially in the developing countries, claiming over 200 000 lives in 2000 [1]. Typhoid fever is a systemic infection caused by *Salmonella enterica* serotype typhi (*S. typhi*). Paratyphoid fever, caused by *Salmonella paratyphi A*, B or C, has a disease presentation highly similar to that of typhoid fever, but, at least in Jakarta, seems to follow a distinct route of transmission: whereas typhoid fever is spread predominantly within the household, paratyphoid fever is mainly transmitted outside the patient's home [2]. The identification of such risk factors and the most relevant route of transmission of the disease are essential for the development

of control strategies and the allocation of public health resources.

Several case-control studies have documented risk factors for typhoid fever at the community level, such as inadequate hygiene, lack of microbiologically safe drinking water or the consumption of street food [2]. Within this environmental context, an individual's genetic makeup may predispose subjects to acquisition of typhoid fever or development of severe disease [3]. For instance, an association between the single nucleotide polymorphism (SNP) *TNFA* -308 and typhoid fever has been reported in Vietnam. Together with HLA-DRB1*0301/6/8 and HLA-DQB1*0201-3, the *TNFA*-308*A allele was thought to be associated with susceptibility to typhoid fever [4,5].

In families with members displaying increased susceptibility to infection with the intracellular pathogens salmonella and mycobacteria, defects in IL-12/IFN γ type-1 cytokine mediated activation of macrophages have been found [6], suggesting that there is considerable overlap in immune responses against these unrelated intracellular bacterial pathogens. Recently, variants in the shared *PARK2* and *PACRG* regulatory region have been found to act as common risk factor for manifest infection by *M. leprae*: a strong association (i.e. OR of 3–5) was demonstrated between the *PARK2*_e01(-2599) polymorphism and leprosy [7]. Mutations in the *PARK2* gene encoding Parkin, have been identified as the cause of autosomal recessive juvenile Parkinsonism [8]. Parkin is a E3 ubiquitin ligase that is required for polyubiquitination of proteins before degradation by the proteasome [9]. Parkin Co-Regulated Gene (*PACRG*) is a reverse strand gene located upstream of the *PARK2* gene. The gene product, termed Glup, forms a large molecular chaperone complex containing heat shock proteins (Hsp) and chaperonin components. Glup binds Parkin via Hsp70, and this multicomponent aggregate may deal with bacterial proteins by breaking them down or turning them into harmless molecules [10]. *PARK2* and *PACRG* share a common promoter regulating their expression [11]. Of interest here, variants in this shared regulatory region have been found to act as a common risk factor for the acquisition of leprosy and there was some evidence that the T allele of this polymorphism acts in a dominant fashion [7].

Given the overlap in immune response to *Salmonellae* and mycobacteria, and its proven role in leprosy, we hypothesized that *PARK2/PACRG* polymorphisms may be associated with clinical typhoid and paratyphoid fever. In addition, *in vitro* studies suggested a possible role for *PARK2/PACRG* regulated genes in *Salmonella* pathogenesis, since they link this pathway to intracellular bacterial evasion mechanisms [12–14]. Parkin, the protein encoded by *PARK2* has ubiquitin ligase (E3) activity [15]. The ubiquitin-proteasome pathway is important in protein processing and degradation, and contributes to quality control of proteins within cells and antigen processing for cross-presentation [16]. Invasion of host cells by *Salmonellae* requires the reversible activation of Cdc42 and Rac1 by bacterial encoded SopE and SptP, which must exert their function at different times during uptake. Although both proteins are delivered into the host cell cytoplasm at approximately equivalent amounts, SopE is rapidly degraded through a proteasome-mediated pathway, while SptP exhibits much slower degradation kinetics. Stabilization of SopE by proteasome inhibition prevents cellular recovery after bacterial infection and therefore continuation of a permissive environment for the bacteria to replicate or evade host defences [12,13]. This mechanism is important in *Salmonella* interaction with its host cells, and we hypothesized that modification of its activity might result in an association between *PARK2/PACRG* polymorphism and typhoid and paratyphoid fever.

Given the above hypothesis on the possible role of ubiquitination and degradation of bacterial proteins in the cellular pathogenesis of these diseases [12–14,16], we investigated the role of *PARK2* and *PACRG* polymorphisms as host-dependent risk factors for acquisition of *Salmonella typhi* and *S. paratyphi* infection.

Materials and methods

Study design

From June 2001 to February 2003 patients with blood culture-confirmed *Salmonella typhi* ($n = 69$) or *Salmonella paratyphi A* ($n = 24$) were identified in a prospective, community-based, case-controlled passive surveillance study in the Jatinegara district of Jakarta, Indonesia [2]. In this surveillance study, we enrolled 1019 consecutive individuals living in the study area who presented with fever lasting ≥ 3 days to one of 24 healthcare facilities in the district. The study was designed to address both environmental [2,17,18] and genetic determinants of susceptibility to enteric fever and was aimed at investigating new associations as well as identifying specific candidate genes that might reveal meaningful immunological insights [19]. Full details of the enrolment of patients have been described elsewhere [2].

Blood cultures were collected into Bactec bottles (aerobic) containing antibiotic absorbing resins (Becton Dickinson, Sparks, MD, USA) that were provided to the centres by the study group free of charge. Every second consecutive fever patient with a negative blood culture, or having a pathogen other than *Salmonella* cultured, was selected as a fever control. Also, during the surveillance community controls were randomly selected within a random household in every third *rukun tetangga* (RT) from a total of 1140 RTs in Jatinegara, RT being the smallest administrative unit of 40–60 households in the area. When a community control reported fever in the 30 days preceding the interview or refused participation, the house on alternating sides of the initially selected household was approached. The selection of both groups of controls was nonmatched for age, sex or neighbourhood to limit selection-bias and prevent overmatching. Four controls from both groups for every case of blood culture-confirmed enteric fever were selected in order to increase the statistical power. Furthermore, between March and October 2003, 4 participating centres and the Medistra Hospital adjacent to the study area contributed another 23 cases of enteric fever (i.e. 21 typhoid fever and 2 paratyphoid fever cases).

All cases and fever controls were visited at home within one month after the febrile episode that led to the blood culture. Community controls were visited randomly throughout the study period. This study was approved by the Indonesian National Institute of Health Research and Development (*LiBANGkes*) and provincial authorities. Written informed consent was obtained from all participants or their guardians.

Household visits and sample collection

Cases, fever controls and randomly selected community controls were interviewed by trained medical school graduates using a validated, standardized questionnaire as described previously [2,20]. Three ml of blood was collected using an EDTA-containing vacutainer system (Becton Dickinson). Blood samples were stored in a cool box until processing in the Biomedical Laboratory, Faculty of Medicine, Catholic University of Atma Jaya. The plasma was separated and erythrocytes were lysed using lysis buffer (155 mM NH₄Cl, 10 mM KHCO₃, 1 mM EDTA, pH = 7.4). White blood cells were washed twice with phosphate buffered saline (PBS) and stored in a freezer until transport to the Laboratory of Infectious Diseases, Leiden University Medical Centre. Genomic DNA was isolated from the cells essentially as described by Sambrook and Russell [21]. Before assay, DNA was diluted to a suitable concentration and stored in microplates at -20 °C.

Single nucleotide polymorphism (SNP) analysis

SNP analysis was performed using polymerase chain reaction (PCR) amplification followed by restriction enzyme digestion. PCRs were performed using 100 ng of genomic DNA, 200 μM of each dNTP, 10 pmol of each primer, 50 mM KCl, 10 mM Tris-HCl (pH 9.0), 1% Triton X-100, 1.5 mM MgCl₂, 0.5 U of Taq DNA polymerase (Promega, Madison, WI, USA) in a total volume of 25 μl. PCR cycling conditions were as follows: 94 °C for 5 min once, 30 cycles of 94 °C for 45 s, annealing temperature for 45 s, 72 °C for 45 s and 72 °C for 7 min once. Primers used for PCR are based on [9] with major modifications for *PARK2_e01* (-697) and *rs1040079*.

Digestion reactions using *Bsu36 I*, *HpyCH4 IV*, *Bcl I* (New England Biolabs, Ipswich, MA, USA) and *Bfm I* (MBI Fermentas, Vilnius, Lithuania) were performed according to the manufacturers protocols.

We chose to study four SNPs within the shared 5' regulatory region of *PARK2* and *PACRG*, *PARK2_e01* (-2599), *PARK2_e01* (-697), *rs1333955* and *rs1040079* that were independently associated with an increased risk of leprosy [7].

The sequence of the primers, annealing temperatures and restriction enzymes used, the length of the products and the type of alleles are given in Table 1.

Statistical methods

Data from the questionnaires and polymorphisms were entered twice using EpiInfo 6-04b software (US Centers for Disease Control and Prevention, Atlanta, GA, USA), validated and imported into SPSS version 11.5 (SPSS Inc, Chicago, IL, USA) for statistical analysis. The Hardy-Weinberg equilibrium of each SNP was checked in the total population and in each group of respondents. For the comparisons of the proportion, either the Pearson's χ^2 test or Fisher's exact test was used.

Results

Study population

Of 1019 consecutive individuals living in the study area who presented with fever lasting ≥ 3 days, 116 individuals were enrolled with enteric fever and 337 as fever controls. In addition, 322 randomly selected community controls were included as detailed previously [2] and in Fig. 1. Of the 116 cases of enteric fever, 90 were caused by *Salmonella typhi* and 26 by *Salmonella paratyphi A*. Sixty-two community controls reported a possible history of enteric fever; in none of them had this past diagnosis been confirmed by (blood) culture.

Demographic background of cases, fever controls and randomly selected community controls have been described elsewhere [2]. In short, the gender distribution of the typhoid and paratyphoid cases was about even (56 female from 116 cases); in the community controls this increased to 177 female from 322 cases. The median age of cases was 20 years (Interquartile range (IQR): 12-26.5) which was similar to that of fever controls, whereas both were significantly lower than that of the random community controls, i.e. 32 years (IQR: 18-49). Of note, the median age of the

Table 1. Tools to investigate *PARK2/PACRG* polymorphisms: primers, annealing temperature, and product length of SNPs.

SNPs	Direction	Primer sequence*	Annealing temperature	Restriction enzyme	Product length	Allele
<i>PARK2_e01</i> (-697)	Forward	ACAGCCGCTCCCGGTGCAC	62 °C	<i>Bsu36 I</i>	Uncut: 292 bp	Allele C
	Reverse	ATGGGCAGAGTACATCACTTG			Cut: 139 and 153 bp	Allele T
<i>PARK2_e01</i> (-2599)	Forward	TTTAGCAGTATAGACTTCTCAGC	60 °C	<i>HpyCH4 IV</i>	Uncut: 102 bp	Allele T
	Reverse	GAGCATGAGGTTGCAATTAAGA			Cut: 45 and 57 bp	Allele C
<i>rs1333955</i>	Forward	TTGGATTTTCAGGATTTTATAGC	58 °C	<i>Bcl I</i>	Uncut: 153 bp	Allele T
	Reverse	CTGCCAGCCAGGTTCTCG			Cut: 66 and 87 bp	Allele C
<i>rs1040079</i>	Forward	CCATGAGTATAGGAGGAACCTGT	52 °C 3x	<i>Bfm I</i>	Uncut: 103 bp	Allele G
	Reverse	GGACTAAAGGGCATGGTGAG	62 °C 25x		Cut: 23 and 80 bp	Allele A

*Primer sequences are based on [9] with major modifications for *PARK2_e01* (-697) and *rs1040079*.

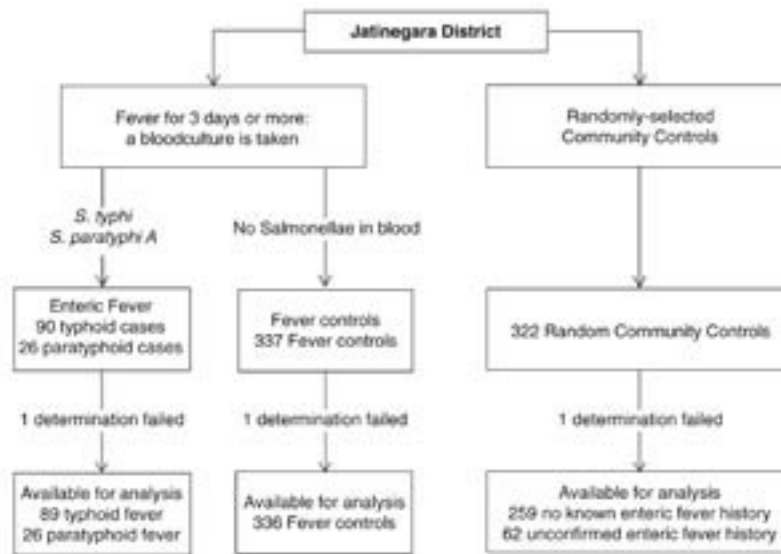


Fig. 1. Flow chart detailing the inclusion of typhoid and paratyphoid fever patients, and fever controls and the randomly selected community controls.

community controls that did not have a history of typhoid or paratyphoid fever was 32.5 years (IQR: 18–50), which was identical to that in community controls with a self-reported history of enteric fever. The age of typhoid fever cases did not differ significantly from that of paratyphoid fever cases. The population of Jatinegara is a mixture of, mainly, Indonesians from different islands of the archipelago and in individual cases it is not possible to designate a subject to one group or to exclude admixture with certainty. However, based on the sublocation in the area and the subjects' names the ethnic makeup of the three study populations did not differ; no stratification with respect to possible admixture was made. In the nonenteric fever control group, patients could be infected with various bacterial and viral pathogens, each having distinct disease mechanisms. Therefore, the underlying genetic susceptibilities for this group could also be diverse. Although the inclusion of a fever control group would not provide a consistent reference group, we decided to present the findings in this group to further illustrate allelic frequencies in the population.

In 1 (1%) of 90 typhoid fever cases, 1 (0.3%) of 337 fever controls and 1 (0.3%) of 322 community controls, we could not determine the SNP alleles due to technical difficulties.

Hardy-Weinberg equilibrium calculation

The genotypes of *PARK2_e01(-697)*, *PARK2_e01(-2599)*, *rs1333955* and *rs1040079* were found to be in Hardy-

Weinberg equilibrium in all cases, fever controls, community controls and in the total group of respondents ($P > 0.7$ in every group and in total for every SNP).

Genotyping PARK2/PACRG SNPs

The genotypic frequencies in cases, fever controls and randomly selected community controls are given in Table 2. In cases, fever controls and community controls alike, TT was found to be the most common genotype for *PARK2_e01(-697)* and *PARK2_e01(-2599)* (62% and 57%, respectively). CC was the most common genotype for *rs1333955* (50%), whereas AA was the most common genotype for *rs1040079* (63%). The most common allele for *PARK2_e01(-697)* and *PARK2_e01(-2599)* is the T-allele (proportion of 79% and 75%, respectively). For *rs1333955*, C is the most common allele with a proportion of 70%. A is the most common allele for *rs1040079* with a proportion of 79%.

In a previous study demonstrating a strong association between the *PARK2_e01(-2599)* polymorphism and leprosy, there was some evidence that the T allele of this polymorphism acts in a dominant fashion. Because only 3 typhoid fever cases and 1 paratyphoid fever case were CC homozygotes, the present study has limited power to test such a hypothesis. Furthermore, the T allele appears to have a higher frequency in the two control populations in this study (75% than in the Vietnamese and Brazilian population (67 and 61%, respectively) [9], possibly reflecting differences in ethnic background.

Table 2. Genotypic frequencies in typhoid and paratyphoid cases, fever controls and randomly selected community controls.

Locus/genotype	Typhoid cases (n = 89)		Paratyphoid cases (n = 26)		Community controls (n = 321)		Fever controls (n = 336)	
	n	%	n	%	n	%	n	%
<i>PARK2_e01(-697)</i>								
CC	6	7	2	8	14	5	16	5
TC	29	33	10	38	110	34	104	31
TT	54	60	14	54	197	61	216	64
<i>PARK2_e01(-2599)</i>								
CC	3	3	1	4	24	7	20	6
TC	29	33	7	27	121	38	124	37
TT	57	64	18	69	176	55	192	57
<i>rs1333955</i>								
CC	49	55	16	61	154	48	167	50
TC	34	38	9	35	139	43	137	41
TT	6	7	1	4	28	9	32	9
<i>rs1040079</i>								
AA	58	65	15	57	203	63	207	62
AG	27	30	9	35	106	33	115	34
GG	4	5	2	8	12	4	14	4

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When comparing the frequencies of these alleles amongst the case group and randomly selected community controls, we found that the frequency of allele T of *PARK2_e01(-2599)* was significantly higher in enteric fever cases ($P = 0.03$). This difference was also significant when we excluded the community controls with an (unconfirmed) history of enteric fever ($P = 0.02$). We did not observe a significant difference in frequency of allele T of *PARK2_e01(-2599)* when we compared fever controls to community controls nor upon comparison of enteric fever cases and fever controls. The allele distribution of the other polymorphisms studied, *PARK2_e01(-697)*, *rs1333955* and *rs1040079* was not significantly different in individuals with enteric fever when compared to those with fever due to other causes or randomly selected community controls.

Odds ratios (OR) were calculated on comparison of the alleles of *PARK2_e01(-697)*, *PARK2_e01(-2599)*, *rs1333955* and *rs1040079* in typhoid fever and paratyphoid fever with randomly selected community controls. Allele T *PARK2_e01(-2599)* was significantly but weakly associated with enteric fever (OR: 1.51, 95%CI: 1.02–2.23). This association became somewhat stronger when we compared the enteric fever cases to community controls without a history of enteric fever (OR: 1.58, 95%CI: 1.06–2.36). For *PARK2_e01(-697)*, *rs1333955* and *rs1040079* polymorphisms, we did not observe an association of a particular allele or genotype with susceptibility to or resistance against typhoid fever and paratyphoid fever grouped as enteric fever, or typhoid fever alone.

Discussion

The main finding of this study is that the common allele T of the *PARK2_e01(-2599)* polymorphism is significantly but weakly associated with typhoid and paratyphoid fever patients as compared to randomly selected community controls. The same polymorphism, i.e. *PARK2_e01(-2599)* within *PARK2* and *PACRG*, was, of all the polymorphisms in this gene region, most strongly associated with clinical leprosy in a Vietnamese population as well as a Brazilian population [7]. Alleles within this gene region that were less strongly but significantly associated with leprosy, i.e. *PARK2_e01(-697)*, *rs1333955* and *rs1040079*, were not found to be associated with typhoid and paratyphoid fever. The findings of the present study therefore, support a role for the *PARK2* and *PACRG* genes in susceptibility to *S. typhi* and *S. paratyphi* as they do, more strongly, in susceptibility to *M. leprae*, and they suggest that the implicated mechanism linked to ubiquitination and proteasome-mediated protein degradation could be a common pathway in the intracellular fate of these intracellular pathogens [6,22].

To study the association of *PARK2/PACRG* polymorphisms and susceptibility to typhoid fever and paratyphoid fever, we compared the prevalence of the polymorphisms in blood culture-confirmed cases of typhoid fever and paratyphoid fever to those of randomly selected community controls from the same study area. Provisions taken to minimize misclassification of cases and controls have been described in detail elsewhere [2]. To get a robust estimate of the prevalence of *PARK2/PACRG* polymorphisms in the population, we included 4 controls for every typhoid or paratyphoid fever patient. Furthermore, fever controls that, similarly to the

cases, presented with ≥ 3 days of fever, but from whom blood cultures showed either no growth or growth of bacteria other than *Salmonellae*, were recruited during the whole study period. Although the fever controls probably suffered from a divergent spectrum of diseases other than enteric fever, and therefore do not constitute a consistent reference group as the random community controls do, we decided to include the findings in this group to further illustrate the allelic frequencies found in the Indonesian population. Nineteen percent of the randomly selected community controls reported a possible episode of typhoid fever in the past. Probably, this percentage is an overestimation of the real number of cases since most fever patients are empirically treated in outpatient clinics without confirmatory diagnosis, but importantly, the distribution of polymorphisms in the community control group was not significantly different when these community controls were left out of the analysis. The age of the typhoid cases and the randomly selected community controls did differ, as the incidence of typhoid is higher in the age group < 20 years. In this respect, however, the distribution of polymorphisms in the community control group did not differ for different age cohorts, e.g. those $< \text{or} > 20$ years old. Of note, local HIV prevalence is low (e.g. 9 HIV positives were detected among 572 TB patients in a parallel study in Jakarta) and is unlikely to be an important confounder.

We hypothesized that *PARK2/PACRG* polymorphisms might be associated with typhoid and paratyphoid fever, given the overlap in immune responses to salmonella and mycobacteria [6], the finding that *PARK2/PACRG* polymorphisms are strongly associated with clinical leprosy [7] and *in vitro* studies that suggest a possible role for *PARK2/PACRG* regulated genes in *Salmonella* pathogenesis and link this pathway to intracellular bacterial evasion mechanisms and antigen processing for cross-presentation [12–14,16]. The study was powered to discern a similar strong association as described for leprosy, i.e. an OR of 3–5. To have the necessary power to confirm that the T allele of the *PARK2_e01* (~2599) polymorphism acts in a dominant fashion, a study with a much larger sample size would be required, because only three typhoid and one paratyphoid case were CC homozygotes. In addition, the T allele appears to have a higher frequency in the Indonesian population (i.e. about 75%) than that previously found in the Vietnamese and Brazilian populations (about 65%) [7]. Given the weak but significant association between a *PARK2* polymorphism and typhoid and paratyphoid fever compared to randomly selected community controls, future studies of larger typhoid cohorts in different populations should elucidate to what extent these processes may play a role in the complex host defence mechanisms against *Salmonella*.

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CHAPTER 5

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