

Metagenomics of Microbial Communities in Gallbladder Bile from Patients with Gallbladder Cancer or Cholelithiasis

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Abstract

Salmonella typhi and Helicobacter infections have been shown to increase risk of gallbladder cancer (GBC), but findings have been inconsistent. Other bacterial infections may also be associated with GBC. However, information on microbial pathogens in gallbladder bile of GBC patients is scarce. We aimed to investigate the microbial communities in gallbladder bile of patients with GBC and cholelithiasis (CL). Seven GBC patients and 30 CL patients were enrolled in this study. Genomic DNA was extracted from bile and the V3-V4 region of 16S rRNA was amplified. The sequencing results were compared with the 16S database, and the bacteria were identified by homology searches and phylogenetic analysis. DNA was detected in the bile of three GBC (42.9%; Bolivia, 1; Chile, 2) and four CL patients (13.3%; Bolivia, 1; Chile, 3). Of the 37 patients, 30 (81.1%) were negative and unable to analyze. Salmonella typhi and Helicobacter sp. were not detected in bile from any GBC patients. As the predominant species, Fusobacterium nucleatum, Escherichia coli, and Enetrobacter sp. were detected in bile from GBC patients. Those in bile from CL patients were Escherichia coli, Salmonella sp., and Enerococcus gallinarum. Escherichia coli was detected in bile samples from both GBC and CL patients. Whether the bacteria detected in bile from GBC patients would associated with the development of GBC warrant further investigation.

Keywords: Microbial pathogens- next generation sequencing- 16S rRNA- V3-V4 region- gallbladder cancer

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Introduction

The important role of cholelithiasis (CL) and the presence of gallstones in the development of gallbladder cancer (GBC) has been established in several previous studies (Randi et al., 2006; Roa et al., 2006; Hsing et al., 2007; Shrikhande et al., 2010; Dutta et al., 2013). Kanthan et al., (2015) divided the risk factors for GBC into four broad categories: patient demographics, gallbladder abnormality, patient exposure, and infections. They concluded that not only gallstones, but also Salmonella and Helicobacter infections, were important risk factors for GBC. Several studies (Kumar et al., 2006; Walawalkar et al., 2013) have demonstrated an association between bacterial infections and increased risk of GBC. However, although associations between Salmonella typhi (Andia et al., 2008; Nagaraja and Eslick, 2014) and Helicobacter (Martel et al., 2009; Pandey et al., 2010; Yakoob et al., 2011) infections and GBC risk have been investigated by many researchers, the findings remain inconsistent. Apparent discrepancies may be explained by differences in the samples used (gallbladder bile, gallbladder tissue,

and serum) and/or in the diagnostic procedures used (culture method, serological examination, and polymerase chain reaction [PCR] analysis). More detailed studies are therefore needed to ascertain if these bacterial infections are indeed related to GBC risk.

A previous study demonstrated that PCR analysis was the most sensitive of the above methods for detecting Salmonella typhi in bile samples (Zhou and Pollard, 2010). Next generation sequencing (NGS) technology can be used to detect and identify microbial organisms directly in the natural environment or in human biological samples, without the need for isolation and confirmation by culture (Salipante et al., 2013). This technology also enables the detection and identification of unculturable microorganisms in biological samples.

We hypothesized that tumours in organs containing body fluid may be caused by microbes in the fluid; more specifically GBC may be caused by Salmonella typhi, Helicobacter sp., or other unknown bacteria in gallbladder bile. Previous studies have investigated the bacterial communities in bile collected from CL patients or healthy individuals using 16S rRNA gene sequencing (Wu et al.,

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2013; Shen et al., 2015; Ye et al., 2016). However, to the best of our knowledge, no previous study has examined the bacteria in gallbladder bile collected from patients with both GBC and CL using NGS technology.

We therefore conducted a preliminary case-control study to reveal the association between bacterial bile infection and GBC risk, and compare gallbladder bile bacteria between patients with GBC and those with CL.

Materials and Methods

Subjects

A total of seven GBC patients (Bolivia, 2; Chile, 5), and 30 CL patients (Bolivia, 22; Chile, 8) were enrolled in this study. Each patient had a diagnosis of GBC or CL at the Instituto de Gastroenterología Boliviano-Japonesa in La Paz, Bolivia, from August 2014 to August 2015, or at the Sotero del Río Hospital in Santiago, Chile, from December 2014 to May 2016.

Bile sample collection

Gallbladder bile samples were collected from all patients at the hospital, after they were diagnosed with GBC or CL. At least 1 mL of bile was collected from each patient by aspiration from the gallbladder after laparoscopic cholecystectomy, injected into a sterile 1.5 mL Eppendorf safe-lock tubes, and covered with a lid. All samples were stored at -80°C and transported from Bolivia or Chile to Japan under frozen conditions. The samples remained stored at -80°C until DNA extraction.

DNA extraction from bile samples

Genomic DNA was extracted from the bile samples using NucleoSpin Soil (Macherey-Nagel GmbH and Co. KG, Duren, Germany). The extracted DNA was purified using an AMPure XP kit (Beckman Coulter, Inc., CA, USA), and the concentrations were quantified using a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific Inc., MA, USA). Double-stranded DNA concentrations were measured using a Quant-iT dsDNA BR Assay Kit (Thermo Fisher Scientific Inc.).

Library preparation

Library preparation was performed according to the instructions (Illumina, 2015) provided by Illumina, Inc. (San Diego, CA, USA). The devices, reagents, and primers used are noted below.

Amplicon PCR

A 460-bp region of DNA was amplified, including the 16S V3-V4 region, using NGS with Tks Gflex DNA Polymerase (Takara Bio Inc., Tokyo, Japan) and a Nextera XT Index Kit (Illumina, Inc.). The 16S amplicon PCR forward and reverse primers were as follows:

341F, 5'-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGCWGCAG-3';

806R, 5'-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTACHVGGGTWCTAAT-3'

The PCR amplification conditions were as follows: 94°C for 60 s for 1 cycle; 98°C for 10 s, 50°C for 15 s, and 68°C for 15 s for 28 cycles; 4°C hold.

PCR clean-up

The 16S V3 and V4 amplicon was purified from free primers and primer dimer species using AMPure XP beads (Beckman Coulter, Inc.).

Index PCR

This step was performed using a Nextera XT Index Kit (Illumina, Inc.). The amplification conditions were as follows: 94°C for 60 s for 1 cycle; 98°C for 10 s, 60°C for 15 s, and 68°C for 15 s for 8 cycles; 4°C hold. The PCR products were purified using AMPure XP beads (Beckman Coulter, Inc.).

Library quantification, normalization, and pooling

The libraries were evaluated using an Agilent 2200 TapeStation (Agilent Technologies, Santa Clara, CA, USA), quantified using a fluorometric method, and diluted with 10 mM Tris pH 8.5 buffer solution to 4 nM. Each 5 μL sample was then pooled for one MiSeq run.

Library denaturing and MiSeq sample loading

Library denaturing was performed using a MiSeq Sequencing System (Illumina, Inc.), MiSeq Reagent Kit v3 (Illumina, Inc.), and Phix Control Kit v3 (Illumina, Inc.). Prior to cluster generation and sequencing, pooled libraries were denatured with NaOH, diluted with hybridization buffer solution, and heat denatured. A minimum of 5% Phix was included in each run.

Metagenomics workflow

The metagenomics was conducted using the following programs, database, and methods.

Clustering: CD-HIT-OUT (ver. 0.0.1). Homology: BLAST (Ver. 2.2.20), DDBJ 16S rRNA database (Ver. 2016_08_17). Systematic taxonomy: RDP classifier (Ver. 2.2), Greengenes (the latest version in Aug of 2016). Phylogenetic trees data preparation: PyNest (Ver. 1.2), FastTree (Ver. 2.1.3), GreenGenes (the latest version). Comparative metagenomics: principal coordinate analysis based on unifracs distance matrix, cluster samples analysis by unweighted pair group method with arithmetic mean method.

Ethics statement

The study procedure was approved by the Ethics Committee of Niigata University of Health and Welfare (No.17540-141030), and was conducted according to the principles expressed in the 1964 Helsinki Declaration. Prior to the start of the study, Bolivian and Chilean researchers informed the subjects of the purpose, procedure, and anticipated results of the study, as well as the risks and benefits. All participants provided informed consent.

Statistical analysis

All statistical analyses were performed using Stata Data Analysis and Statistical Software (STATA 14; StataCorp, College Station, TX, USA). Differences between two groups (GBC and CL, Bolivia and Chile) were analysed by χ^2 or Fisher's exact tests. A p value < 0.05 (two-tailed) was considered to indicate statistical significance.

Table 1. Characteristics of Bolivian and Chilean Patients with Gallbladder Cancer

Country Gender	Age, y	Histological diagnosis	Presence of gallstones	Type of cholecystitis	Bacteria in the bile
Bolivia					
Woman	51	Poorly differentiated invasive adenocarcinoma	Positive	Chronic	Negative
Man	45	Bile duct invasive adenocarcinoma	Positive	Chronic	Positive
Chile					
Woman	53	Adenosquamous carcinoma	Positive	Negative	Negative
Woman	68	Poorly differentiated tubular adenocarcinoma	Positive	Acute on chronic	Positive
Woman	71	Poorly differentiated adenocarcinoma	Positive	Chronic	Positive
Man	52	Poorly differentiated carcinoma with signet cells component	Positive	Chronic	Negative
Man	68	Moderately differentiated tubular adenocarcinoma	Negative	Chronic	Negative

Results

The characteristics of seven patients with GBC from Bolivia and Chile are shown in Table 1. Two Bolivian patients (1 woman, 1 man) with GBC were enrolled (mean age 48.0 years), both of whom had gallstones and chronic cholecystitis according to histopathological diagnosis. Only the male patient had bacteria in the bile, giving a detection rate of bile bacteria of 50% (1/2). Five Chilean patients (3 women, 2 men) were enrolled in this study (mean age 62.4 years), four of whom had gallstones. Four patients had chronic cholecystitis, and bile bacteria were detected in two female patients, giving a detection rate of 40% (2/5).

The characteristics of the Bolivian and Chilean patients with CL are shown in Table 2. There were no significant differences in gender, mean age, presence of cholecystitis, rate of cholesterol gallstones, or bile-bacteria-detection rate between Bolivia and Chile.

The detection rates of bile bacteria in Bolivian and Chilean patients with GBC and CL are shown in Table 3. The bile-bacteria-detection rates among Bolivian patients were 50% (1/2) for GBC patients and 14% (3/22) for CL patients, with no significant difference in bile-bacteria-detection rates between the GBC and CL patients ($p = 0.31$). The detection rates in the Chilean patients were 40% (2/5) for GBC patients and 13% (1/8) for CL patients, with no significant difference in bile-bacteria-detection rates between the GBC and CL patients ($p = 0.51$). Bolivian and Chilean patients with

GBC and CL thus had similar detection rates of bile bacteria.

The bacteria detected in bile samples of Bolivian patients with GBC and CL are shown in Table 4. A total of 24 and 12 types of bacteria were found in bile from GBC and CL patients, respectively. The predominant species were *Fusobacterium nucleatum* subsp. *animalis* ATCC 51,191 (*Fusobacterium nucleatum*) in GBC patients and *Escherichia coli* (*E. coli*), *Enterococcus gallinarum*, and *Salmonella* sp. in CL patients. *E. coli*, bacterium 28W412, and *Pseudomonas pseudoalcaligenes* were found in both GBC and CL patients.

Bacteria detected in bile samples from Chilean patients with GBC and CL are shown in Table 5. A total of eight and two types of bacteria were found in the bile from GBC and CL patients, respectively. The predominant species were *E. coli* and *Enterobacter* sp. B10 (2014) in GBC patients, and *E. coli* in the CL patient. *E. coli* was detected in bile samples from both GBC and CL patients. *Enterobacter* sp. B10 (2014) and *Klebsiella oxytoca* were detected in bile samples from two GBC patients.

Discussion

The results of this study demonstrated bacterial infection rates in bile of 42.9% in combined Bolivian and Chilean patients with GBC, and 13.3% in patients with CL. *Salmonella* sp. and *Helicobacter* sp., previously suggested to be associated with the development of GBC,

Table 2. Characteristics of Bolivian and Chilean Patients with Cholelithiasis

	Bolivia (n = 22)	Chile (n = 8)	P value
Gender			
Women (%)	12 (55)	7 (88)	0.2
Men (%)	10 (45)	1 (12)	
Mean Age			
Women (SD)	49.4 (6.1)	55.4 (14.2)	0.32
Men (SD)	44.9 (5.8)	45	
Total (SD)	47.1 (6.2)	54.3 (13.5)	0.054
Cholecystitis (%)	22 (100)	8 (100)	1
Cholesterol gallstone (%)	12 (55)	3 (38)	0.45
Bacteria detected bile (%)	3 (14)	1 (13)	1

Table 3. Detection Rates of Bile Bacteria in Bolivian and Chilean Patients with Gallbladder Cancer and Cholelithiasis

	Positive (%)	Negative (%)	OR	95% CI	P value
Bolivia					
CL	3 (14)	19 (86)	1		
GBC	1 (50)	1 (50)	6.3	0.5-80.3	0.31
Chile					
CL	1 (13)	7 (87)	1		
GBC	2 (40)	3 (60)	4.7	0.4-50.1	0.51
Total					
CL	4 (13)	26 (87)	1		
GBC	3 (43)	4 (57)	4.9	0.9-28.2	0.11

CL, cholelithiasis; GBC, gallbladder cancer; OR, odds ratio; CI, confidence interval; Infection rates are shown in parentheses

Table 4. Bacteria Detected in Bile of Bolivian Patients with Gallbladder Cancer or Cholelithiasis

Consensus Lineage	GBC	CL-1	CL-2	CL-3	#OTU ID
<i>Escherichia coli</i>	+	++		+	Cluster 0
<i>Enterococcus gallinarum</i>				++	Cluster 1
<i>Salmonella</i> sp. ATK1		+	++		Cluster 2
<i>Bacteroides fragilis</i>	++				Cluster 3
<i>Fusobacterium nucleatum</i> subsp.*	+				Cluster 4
<i>Pyramidobacter piscolens</i>	+				Cluster 5
bacterium NLAE-zl-H528	+				Cluster 6
<i>Odoribacter splanchnicus</i> DSM 20712	+				Cluster 7
<i>Anaeroglobus</i> sp. S4-A15	+				Cluster 8
bacterium IARFR1475	+				Cluster 9
<i>Streptococcus</i> sp. ChDC B623	+				Cluster 10
Veillonellaceae bacterium oral taxon 150	+				Cluster 1
<i>Mythylobacterium zatmanii</i>		+	+		Cluster 12
<i>Clostridium</i> sp.	+				Cluster 13
<i>Selenomonas sputigena</i>	+				Cluster 14
<i>Enterococcus faecalis</i>				+	Cluster 15
<i>Bacteroides uniformis</i>	+				Cluster 16
bacterium NLAE-zl-H504	+				Cluster 17
bacterium 28W412	+	+	+		Cluster 18
<i>Clostridium perfringens</i>				+	Cluster 19
*** no hit ***	+	+	+		Cluster 20
<i>Dialister pneumosintes</i>	+				Cluster 21
<i>Selenomonas artemidis</i>	+				Cluster 22
<i>Klebsiella</i> sp.		+	+		Cluster 23
<i>Agrobacterium larrymoorei</i>		+			Cluster 24
<i>Prevotella denticola</i>	+				Cluster 25
bacterium PIC8	+				Cluster 26
<i>Pseudomonas pseudoalcaligenes</i>	+	+	+		Cluster 27
<i>Pseudomonas koreensis</i>	+	+			Cluster 28
<i>Slackia</i> sp. CM382	+				Cluster 29
<i>Bacteroides salyersiae</i>	+				Cluster 30

Table 5. Bacteria Detected in Bile of Chilean Patients with Gallbladder Cancer or Cholelithiasis

Consensus Lineage	GBC-1	GBC-2	CL	#OTU ID
<i>Escherichia coli</i>		++	++	Cluster 0
<i>Enterobacter</i> sp. B10 (2014)	++	+		Cluster 1
<i>Klebsiella oxytoca</i>	+	+		Cluster 2
bacterium NLAE-zl-P344		+		Cluster 3
<i>Streptococcus sanguinis</i>			+	Cluster 4
<i>Enterococcus durans</i>		+		Cluster 5
<i>Propionibacterium acidifaciens</i>		+		Cluster 6
<i>Klebsiella pneumoniae</i>		+		Cluster 7
<i>Citrobacter</i> sp. AL7	+			Cluster 8

OTU, an operational taxonomic unit; ++, predominant species; +, detected species.

were not detected in the bile of GBC patients, while *Fusobacterium nucleatum*, *E. coli*, and *Enterobacter* sp. were the predominant species in Bolivian and Chilean GBC patients.

Gallstones with attendant chronic cholecystitis has been reported to be an important risk factor for GBC (Hundal and Shaffer, 2014). However, although several studies have examined the role of bacterial infection in GBC risk, inconsistent findings have been reported. There are several possible explanations for the apparent discrepancy in results regarding the relationship between bacterial infection and GBC risk. The use of different samples and methods may have led to differences among results. There are various ways of checking for bacterial infections, e.g., culture methods, serological examination, and PCR, of which PCR has been reported to be the most sensitive for detecting bacteria in bile samples (Zhou and Pollard, 2010). Culture methods are commonly used to detect bacterial infection, but specific media are needed to allow the development of anaerobic or acid-fast bacteria, making it difficult to culture and identify multiple types of bacteria simultaneously. NGS-based 16S rRNA sequencing has recently been developed and used for microbiological research. This technology provides a

culture-free method that permits the analysis of the whole microbial community in a sample (Salipane et al., 2013). Previous studies have mainly examined the associations between *Salmonella typhi* or *Salmonella paratyphi* (Andia et al., 2008; Nagaraja and Eslick, 2014), or *Helicobacter sp.* (Martel et al., 2009; Pandey et al., 2010; Yakoob et al., 2011) infections and GBC risk, but other unknown bacteria may also be related to GBC risk. We therefore aimed to clarify the characteristics of the entire microbial communities in gallbladder bile from GBC and CL patients using NGS-based 16S rRNA sequencing.

Previous studies found detection rates of 44.8% (Roa et al., 1999) and 81.0% (Csendes et al., 1994) for bile bacteria in Chilean patients with GBC, compared with a detection rate of 40.0% in the current study, using NGS technology. The reasons for the lower detection rate were unclear, but it is possible that the chance of bacterial infection may have decreased in recent years as a result of antibiotic administration. The predominant species of bile bacteria in Bolivian GBC patients was *Fusobacterium nucleatum*, which is known to be isolated from the oral cavity (Gharbia et al., 1990). This bacterium is known to be an opportunistic pathogen, but since 2011, many researchers has reported to be associated with colorectal cancer risk (Ray, 2011; Castellarin et al., 2012; Keku and McCoy, 2013; Miwa et al., 2015; Noshro et al., 2016; Repass et al., 2016). Primary sclerosing cholangitis and ulcerative colitis (UC) patients are recognized as a high-risk group for cholangiocarcinoma and GBC (Herzog and Goldblum, 1996; Yamamoto et al., 2003; Lewis et al., 2007; Noda et al., 2009; Pastogi et al., 2012). *Fusobacterium* and other bacteria were detected in the UC patients (Andoh et al., 2007). Our finding that *Fusobacterium nucleatum* was detected in the bile from the GBC patients might be explained within the association among GBC risk, UC, and *Fusobacterium* infection. In contrast, the predominant bacteria of Chilean GBC patients were *E. coli* and *Enterobacter sp. B10* (2014). Given that these bacteria were also found in previous studies in Chile (Csendes et al., 1994; Roa et al., 1999), the bacteria may live in the bile of some GBC patients. A previous study has demonstrated that *Fusobacterium nucleatum*, *E. coli*, and *Enterobacter sp.* infections contribute to promoting colon cancer (Gagnaire et al., 2017). The presence of these bacteria in the bile might also increase the risk of GBC by the same mechanism as colon cancer. However, further studies are needed to confirm the relationship between these bacterial infections and the development of GBC.

Contrary to our expectations, *Salmonella sp.* was not detected in bile from the patients with GBC in the present study, though this may have been because of the small number of Bolivian GBC patients. On the other hand, the failure to detect *Salmonella sp.* in the bile of Chilean patients with GBC may be related to a decrease in infection rate of *Salmonella typhi*. Typhoid fever is known to be caused by *Salmonella typhi*, and was a common infection in Chile with an incidence of 100–121 per 100,000 population from 1976–1985. The incidence rate subsequently decreased sharply to the current status of 3.3 per 100,000 population years (Andia et al., 2008). The lack of *Salmonella sp.* detection in the Chilean GBC

patients may thus reflect the decreased infection rate of *Salmonella typhi*. Furthermore, most Chilean patients with GBC were diagnosed at an advanced stage, and bile collection from the gallbladder was thus difficult and the collected volume was small. *Salmonella typhi* infection prior to GBC diagnosis was treated with antibiotics, which may thus have affected the development of the bacteria. A report from India, which has a higher incidence of GBC in the north compared with the south, found higher *Salmonella typhi* infection rates in the north (Banerjee et al., 2014). The detection rate of *Salmonella sp.* from the bile might thus parallel the *Salmonella typhi* infection rate among people living in high-GBC-incidence areas.

Nine studies have reported on the associations between *Helicobacter* infection and GBC risk since 2000 (Martel et al., 2009; Pandey et al., 2010; Yakoob et al., 2011), of which eight detected *Helicobacter sp.* in bile or gallbladder tissue from GBC patients. These nine studies used various different methods (PCR, serological examination, and culture) to detect *Helicobacter sp.*, including PCR in three, PCR or serological examination in four, serological examination in one, and all three methods in one. A study in Germany using all three methods failed to detect *Helicobacter sp.* (Bohr et al., 2007), and the authors suggested that these negative results may reflect the low incidence of GBC in Germany. Overall, these studies suggest that *Helicobacter* infection may be related to the development of GBC, particularly in areas of high GBC incidence.

To the best of our knowledge, no previous study has examined the association between *Helicobacter* infection and GBC risk in Chileans. We found no *Helicobacter* in any bile samples from Bolivian or Chilean patients with GBC or CL. However, Fox et al., (1998) detected *Helicobacter sp.* in bile and gallbladder tissue from Chilean patients with cholecystitis using PCR. The detected bacteria were *Helicobacter bilis*, *Flexispira rappini* (ATCC 49317), and *Helicobacter pullorum* based on the sequencing data. They suggested an association between bile-resistant *Helicobacter* infections and gallbladder disease, and pointed out the need to confirm the roles of these bacteria in the development of GBC. Chile has a high incidence of GBC, and further studies are therefore needed to clarify the association between *Helicobacter* infection and GBC. Wang et al., (2015) reported that *Helicobacter pylori* was rapidly induced into *Helicobacter pylori* L-form in human bile, which is difficult to isolate using routine bacteriological methods and difficult to identify even by PCR. It may therefore be necessary to pay specific attention to the L-form, as well as wild-type *Helicobacter sp.* in order to increase its detection in bile.

This study had some limitations. The sample sizes, especially in Bolivian GBC patients and Chilean patients with GBC and CL, were small, and the low bacterial detection rates in the bile may not reflect the accurate detection rates. Furthermore, the absence of a significant difference in bacterial infection rates between GBC patients and CL patients may also have been caused by a lack of statistical power associated with the small number of patients analysed. This is reflected by the wide 95% CI. More bacterial strains were detected in bile from

Bolivian compared with Chilean patients. The reasons for this difference are unclear, but may be related to differences in ethnicity or daily life environments between the populations. Despite these limitations, this study provides the first evidence regarding the characteristics of the microbial communities in bile from Bolivian and Chilean patients with GBC or CL using NGS technology.

In summary, we clarified the nature of the microbial communities present in bile in Bolivian and Chilean patients with GBC and CL using 16S rRNA metagenomics. The predominant species in Bolivian GBC patients was *Fusobacterium* sp., but those in Chilean GBC patients were *E. coli* and *Enterobacter* sp. B10 (2014), while *Salmonella* sp. and *Helicobacter* sp. were not detected in bile from GBC patients. These findings provide fundamental data regarding the pathogenesis of GBC in Bolivia and Chile, but further studies are needed to ascertain the roles of the bacteria detected in the present study in the development of GBC.

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