(1→3)- β -D-GLUCAN AND GALACTOMANNAN FOR DIFFERENTIATING CHEMICAL “BLACK PARTICLES” AND FUNGAL PARTICLES INSIDE PERITONEAL DIALYSIS TUBING

Asada Leelahavanichkul,¹ Krit Pongpirul,^{2,3} Nisa Thongbor,⁴ Navaporn Worasilchai,¹ Kwanta Petphrak,⁵
Bussakorn Thongsawang,⁵ Piyaporn Towannang,⁶ Pichet Lorvinitnun,⁴ Kanya Sukhontasing,¹
Pisut Katavetin,^{6,7} Kearkiat Praditpornsilpa,⁷ Somchai Eiam-Ong,⁷ Ariya Chindamporn,¹
and Talerngsak Kanjanabuch⁵⁻⁷

Departments of Microbiology¹ and of Preventive and Social Medicine,² Faculty of Medicine, Chulalongkorn University, Bangkok, Thailand; Department of International Health,³ Johns Hopkins Bloomberg School of Public Health, Baltimore, Maryland, USA; Sappasit Prasong Hospital,⁴ Ubon Ratchathani; Kidney and Metabolic Research Unit,⁵ Faculty of Medicine, Chulalongkorn University, Bangkok; Peritoneal Dialysis Excellent Center,⁶ King Chulalongkorn Memorial Hospital, Bangkok; and Division of Nephrology,⁷ Department of Medicine, Faculty of Medicine, Chulalongkorn University, Bangkok, Thailand

◆ **Background:** Aseptic, sheet-like foreign bodies observed inside Tenckhoff (TK) catheter lumens (referred to as “black particles”) are, on gross morphology, hardly distinguishable from fungal colonization because these contaminants adhere tightly to the catheter. Detection of fungal cell wall components using (1→3)- β -D-glucan (BG) and galactomannan index (GMI) might be an alternative method for differentiating the particles.

◆ **Methods:** Foreign particles retrieved from TK catheters in 19 peritoneal dialysis patients were examined microscopically and cultured for fungi and bacteria. Simultaneously, a Fungitell test (Associates of Cape Cod, Falmouth, MA, USA) and a Platelia *Aspergillus* ELISA assay (Bio-Rad Laboratories, Marnes-La-Coquette, France) were used to test the spent dialysate for BG and GMI respectively.

◆ **Results:** Of the 19 patients, 9 had aseptic black particles and 10 had fungal particles in their tubing. The fungal particles looked grainy, were tightly bound to the catheter, and appeared more “colorful” than the black particles, which looked sheet-like and could easily be removed by milking the tubing. Compared with effluent from patients having aseptic particles, effluent from patients with fungal particles had significantly higher levels of BG (501 $\pm$ 70 pg/mL vs. 46 $\pm$ 10 pg/mL) and GMI (10.98 $\pm$ 2.17 vs. 0.25 $\pm$ 0.05). Most of the fungi that formed colonies inside the catheter lumen were molds not usually found in clinical practice, but likely from water or soil, suggesting environmental contamination. Interestingly, in all 10 patients with fungal colonization, visualization of black particles preceded a peritonitis episode and TK catheter removal by approximately 1–3 weeks; in patients with aseptic particles, a 17-week onset to peritonitis was observed.

◆ **Conclusions:** In all patients with particle-coated peritoneal dialysis tubing, spent dialysate should be screened for BG and GMI. Manipulation of the TK catheter by squeezing, hard flushing, or even brushing to dislodge black particles should be avoided.

Correspondence to: T. Kanjanabuch, Division of Nephrology, Department of Medicine, Faculty of Medicine, Chulalongkorn University, Bangkok 10330 Thailand.
golfnephro@hotmail.com

Received 10 September 2014; accepted 17 August 2015.

Replacement of the TK catheter should be suspended until a cause for the particles is determined.

Perit Dial Int 2016; 36(4):402–409 epub ahead of print: 02 Nov 2015
<http://dx.doi.org/10.3747/pdi.2014.00235>

KEY WORDS: Black particles; (1→3)- β -D-glucan; galactomannan; fungal colonization; fungal peritonitis; CAPD.

Since 2008, peritoneal dialysis (PD) has been mandated as a primary mode of renal replacement therapy for end-stage renal disease patients under the “PD first” policy of the Thai Universal Coverage scheme (1). During 2008–2011, our group reported an outbreak in Thailand of dark particles inside the lumens of Tenckhoff (TK) catheters and transfer sets, then called “black particles” (2,3). That dark-colored, sheet-like material, which consisted of several inorganic compounds (calcium phosphate, silicone polymer, and potassium iodide) and organic materials (cell debris and amine proteins), can still be found in PD patients, having an approximate annual incidence of 57.6 per 1000 (2). A definite cause for the black particles remains inconclusive, but the main hypothesis is contamination with iodine-based disinfectant during a transfer set change, or when PD starts, or from a leaking catheter cap. However, a reduction in the use of iodine disinfectant yielded only marginal success in lowering the particle incidence. The presence of black particles was considered harmless because no direct clinical consequences had been demonstrated, at least in short-term follow-up (2).

Fungi are organisms that can grow rapidly in high-glucose conditions, including in the PD environment. Fungi can potentially invade the PD system by several routes (4):

- Gastrointestinal: transluminal penetration by an overgrowth of intraluminal *Candida* species after a period of broad-spectrum antibiotic administration

- Periluminal: downward peri-catheter migration of resident pathologic microflora at the exit site
- Intraluminal: environmental contamination during a bag exchange

Given high atmospheric humidity, the prevalence of fungal infection in tropical regions is relatively higher than it is in other regions (5,6). Some fungi can colonize the inside of the silicone TK tubing, mimicking black particles, particularly in gross morphology, and at an early stage of colonization, they provide no clinical or laboratory clues of peritonitis (7–11). Because of tight adherence to the tubing, the particles cannot be examined directly without tube removal, and so the detection of fungal cell-wall components such as (1→3)- β -D-glucan (BG) and the galactomannan index (GMI) are potentially useful methods for differentiating fungal from nonfungal particles (12,13).

Both of the foregoing biomarkers have been approved by the US Food and Drug Administration for the diagnosis of invasive aspergillosis (14–17). Serum BG higher than 60 pg/mL and a GMI higher than 0.5 support a diagnosis of invasive fungal infection, especially in patients with neutropenia and hematologic malignancy (13,17). Although BG and GMI are not approved for testing PD effluent (PDE) samples, high effluent BG has been reported in a PD patient with fungal peritonitis (18), and findings from our recent study supported the use of BG and GMI in PDE as surrogate biomarkers for a diagnosis of fungal peritonitis (19). The present study is a proof-of-concept pilot to explore whether detection of BG and GMI can be an alternative method for differentiating black particles.

METHODS

PATIENTS

Our study includes 19 PD patients from King Chulalongkorn Memorial Hospital, Bangkok; Sunpasitthiprasong Hospital, Ubon Ratchathani; Roi Et Hospital, Roi Et; Saraburi Hospital, Saraburi; Uttaradit Hospital, Uttaradit; and Buddhasothorn Hospital, Chachoengsao, who, during September 2013 to June 2014, underwent removal of a TK catheter that contained intraluminal foreign particles. Immediately before the operation, a PDE cell count, Gram stain, KOH smear, and culture were performed. The removed catheters were submitted for scanning electron microscopy, direct exam, and standard swab culture. The patients were divided into two groups (aseptic black particles and fungal particles) based on the culture results and direct microscopic examination of the particles (Table 1).

At the time of catheter removal, PDE was collected in BG-free containers and held at -80°C until analyzed. Patient demographics and clinical characteristics, including effluent leukocyte count and culture results at the time when the foreign particles were first observed inside the TK catheter lumen, were retrospectively collected from medical records. Peritonitis was diagnosed according to the International

Society for Peritoneal Dialysis 2010 recommendation [2 of 3 criteria present: signs or symptoms of peritonitis; elevated leukocyte count (≥ 100 cells/ μL), with at least 50% neutrophils; and positivity for pathologic micro-organisms] (20). In addition, cross-sectional measurements of BG and GMI were obtained at the time that foreign-body particles were recognized in 3 asymptomatic patients with clear PDE outside the observational period of the study.

SAMPLE ASSESSMENTS

Foreign particles were examined using KOH, acid-fast, and Gram stain; they were also directly plated onto several culture media, including blood agar, MacConkey agar, chocolate agar, and Sabouraud dextrose agar. The micro-organisms were identified based on characteristics in the region of internal transcribed spacer.

The presence of BG was assessed by an indirect method (Fungitell: Associates of Cape Cod, Falmouth, MA, USA) that detects coagulation of the susceptible horseshoe crab's limulus lysate because of a cascade activation of factor G from the presence of BG, which in turn cleaves the inactive pro-clotting enzyme into the active form and creates a chromophore that absorbs light at 405 nm. The PDE (5 μL) was transferred into test wells in duplicate and was examined according to the manufacturer's protocol.

A sandwich ELISA was used to detect galactomannan (Platelia *Aspergillus*: Bio-Rad Laboratories, Marnes-La-Coquette, France). The PDE (300 μL) was transferred into pre-coated anti-galactomannan ELISA kits according to the manufacturer's protocol. Results are expressed as a ratio to the standard control provided in the kit and are presented as an index of positivity (GMI). Currently, effluent BG exceeding 60 pg/mL and GMI exceeding 0.5 were used as the cut-off for fungal identification.

Analyses of the components of the aseptic black particles used staining (hematoxylin and eosin, Masson trichrome) and scanning electron microscope examination (JSM-5400: Jeol, Tokyo, Japan).

STATISTICAL ANALYSIS

Data were analyzed using descriptive statistics. Comparisons of continuous variables between groups used the Student *t*-test. Any *p* value less than 0.05 was considered significant. All calculations were performed using the SPSS software application (version 11.5: SPSS, Chicago, IL, USA).

RESULTS

PATIENT CHARACTERISTICS AND PDE ANALYSIS

Based on findings from the microscopic examination and particle culture, 9 patients were found to have aseptic particles, and 10 patients were found to have fungal particles without a concomitant bacterial infection. Demographics and clinical characteristics of the patients in the two groups,

TABLE 1
Patient Characteristics and Dialysate Analysis

Variable	Particle type		<i>p</i> Value
	Black	Fungal	
Patients (<i>n</i>)	9	10	
Mean age (years)	57±11	54±11	0.62
Sex [<i>n</i> (%) men]	4 (44)	6 (60)	0.49
Cause of ESRD (<i>n</i>)			
Diabetes mellitus	3	5	0.49
Hypertension	2	3	0.51
Primary glomerular diseases	2	2	0.63
Unknown	2	0	0.18
Indication for Tenckhoff catheter removal (<i>n</i>)			
Living-related kidney transplantation	3	0	NC
Mechanical failure	4	0	NC
Ultrafiltration failure	2	0	NC
Fungal peritonitis	0	9	NC
Sepsis	0	1	NC
Mean duration of PD (months)	24±11	14±16	0.047
Previous bacterial peritonitis (episodes per year)	0.67±0.71	0.88±0.64	0.53
Presence of peritonitis (<i>n</i>)			
At time of particle recognition	0	6	NC
At Tenckhoff catheter removal	0	10	NC
Peritonitis symptoms [<i>n</i> (%)]			
Fever	0	4 (40)	NC
Abdominal pain	0	5 (50)	NC
Cloudy dialysate	0	10 (100)	NC
Mean dialysate leukocyte count (cells/μL)			
At time of particle recognition	14±6	93±58	0.0002
At Tenckhoff catheter removal	23±13	362±31	0.006
Interval between particle recognition and Tenckhoff catheter removal (weeks)			
Median	12	0	<0.0001
Range	8–62	0–13	
Serum albumin (g/dL)	3.1±0.9	2.9±0.9	NC
Dialysate analysis ^a			
(1→3)-β-D-glucan (pg/mL)	43±11	501±70	<0.0001
Galactomannan index	0.26±0.05	10.98±2.17	<0.0001

ESRD = end-stage renal disease; PD = peritoneal dialysis; NC = not calculated.

^a At Tenckhoff catheter removal.

including history of previous bacterial peritonitis, were comparable (Table 1). The patients who had fungal particles had been on PD for a shorter duration, approximately 10 months (24 ± 11 months vs. 14 ± 16 months).

Almost half the patients with fungal colonization (4 of the 10) had no clinical signs or symptoms of peritonitis and a leukocyte count of less than 100 cells/μL when the particles were first recognized. Because none of those patients had a positive fungal culture (although a milking maneuver had been attempted), a provisional diagnosis of aseptic black particles was made at first observation. The other 6 patients presented with concurrent peritonitis and fungal colonization (Table 2), and within the next 48 hours, 5 of those 6 patients had a positive fungal culture from PDE that warranted prompt administration of appropriate antifungal agents. One patient whose blood cultures and cloudy PDE

were initially negative subsequently had a positive culture for *Scedosporium* species based on the particles retrieved from the removed TK catheter. Unfortunately, that patient died of severe sepsis, which might be partly be a result of delayed antifungal administration.

All patients with fungal colonization eventually showed overt peritonitis, with some combination of cloudy dialysate (*n* = 10), fever (*n* = 4), and abdominal pain (*n* = 5), and all finally underwent TK catheter removal. Interestingly, the interval between the detection of foreign particles and the subsequent TK catheter removal was significantly shorter in the group having fungal particles than in the group having black particles (2 ± 1 weeks vs. 17 ± 6 weeks). The dialysate leukocyte count increased from 93 ± 58 cells/μL (range: 12–164 cells/μL) at the initial time of particle recognition to 362 ± 319 cells/μL (range: 135–1050 cells/μL) at time of the catheter removal.

TABLE 2
Characteristics of the Fungal Colonies

Particle color	Organism	(1→3)-β-D-glucan (pg/mL)	Galactomannan index	Time of particle recognition with respect to peritonitis (weeks)	Outcome
Black	<i>Alternaria</i> spp.	≥523	12.54	Concurrent	HD
Black	<i>Scedosporium</i> spp.	302	10.15	Concurrent	HD
Black	<i>Scedosporium</i> spp.	≥523	12.32	Concurrent	HD
Black	<i>Scedosporium</i> spp.	≥523	12.26	3	Death
Black	<i>Cladosporium</i> spp.	≥523	12.23	13	HD
Gray	<i>Simplicillium obclavatum</i>	≥523	6.21	5	HD
Gray	<i>Fusarium</i> species	≥523	10.14	2	HD
Gray	<i>Fusarium</i> species	≥523	12.32	Concurrent	HD
White	Non-albicans <i>Candida</i>	≥523	0.46	Concurrent	HD
White	Non-albicans <i>Candida</i>	≥523	0.41	Concurrent	HD

HD = hemodialysis.

Most of the PD patients with aseptic black particles had no serious infectious complications. All 9 underwent TK catheter removal because of noninfectious indications such as mechanical complications, ultrafiltration failure, or kidney transplantation (Table 1).

FOREIGN PARTICLE CHARACTERISTICS AND EFFLUENT BG AND GMI

Macroscopically, both types of particles were usually dark in color ($n = 17$) and varied in size (ranging from tiny dots to continuous sheets covering the luminal surface of the catheter); however, the particles demonstrated several differing characteristics (Table 3).

Most of the aseptic particles looked initially like color stains or sheet-like material on the luminal surface of the catheter, usually located in the proximal end of the tubing, just above the exit site. At later stages, the sheets became more particle-like and fragile, especially when hard pressure was applied on the tubing. Sometimes, particles would spill out through the transfer set and be collected in drained dialysate. The particles were brown to black in color only [Figure 1(A-E)]. Under direct microscopic analysis, a crystal-like structure [Figure 2(A1)] or amorphous material, or both, were identified. Although the former could be inorganic material as mentioned in a previous publication (2), the latter was an admixture of protein (as revealed by hematoxylin and eosin staining) and cell debris morphologically resembling mesothelial cells [Figure 2(A2)]. Thus, the aseptic particles might be a response of mesothelial cells to chemical reactions.

On the other hand, fungal particles showed discrete cobblestone or sand-like patterns [Figure 2(B1)]. Normally, colonies from a single catheter appeared to be of one color only, unless there was a mixed infection with more than one species. However, various shades of the same color could be found [Figure 1(F-J)]. Most fungal particles in our series were black ($n = 5$), followed by grey ($n = 3$) and white ($n = 2$; Table 2). All of the particles adhered tightly to the silicone tubing and

TABLE 3
Characteristics of Aseptic Black and Fungal Particles

Characteristic	Particle type	
	Black ($n=9$)	Fungal ($n=10$)
Size	Varies	Varies
Shape and texture	Continuous, sheet-like, flat	Skip lesion, convex, oval, cobblestone-like
Color	Black or brown	Varies ^a
Stickiness	Possible to milk out	Tightly adheres to tube, not possible to milk out
Microscopic features	Hard granule or soft proteinaceous material	Filamentous mold or yeast
(1→3)-β-D-glucan and galactomannan index	Undetectable or low	High

^a Usually the same color in one Tenckhoff catheter.

could hardly be moved in a milking maneuver. Hence, the particles could be collected only after the catheter was removed.

Where fungal particles were present, effluent BG (501 ± 70 pg/mL vs. 46 ± 10 pg/mL) and GMI (10.98 ± 2.17 vs. 0.25 ± 0.05) were significantly higher than they were in the presence of aseptic particles. Interestingly, assays of effluent BG and GMI were highly positive in fungal colonization (Figure 3), but GMI was positive at a low level in 2 patients with white fungal particles (later identified as non-albicans *Candida*), possibly because of a limitation of the GMI assay for yeast detection (13,21,22). Moreover, outside the study, an additional 3 asymptomatic patients presented with an

Figure 1 — Tenckhoff catheters with (A–E) aseptic black particles and (F–J) fungal colonization. The pictures were taken before catheter removal.

incidental finding of intraluminal foreign particles and clear PDE (Table 4). Although the particles in those cases adhered tightly to the TK catheter lumen, they were retrieved by shortening the catheter tip or by removing the transfer set. Despite clear PDE, a low PDE cell count, and a negative PDE culture for fungi, effluent BG and GMI levels in the patients were high, with subsequent positive detection of fungi during direct KOH examination and DNA sequencing of the particles (Table 4). The latter findings support the utility of BG and GMI in PDE as indexes of fungal colony presence, regardless of peritonitis.

FUNGUS ISOLATION AND TREATMENT OUTCOMES

Organisms identified from black and grey particles were molds. *Alternaria* species, *Cladosporium* species, and *Scedosporium* species were isolated from black particles; *Simplicillium obclavatum* and *Fusarium* species were identified from gray particles (Figure 4). Only yeast, non-albicans *Candida*, was identified from white particles. In patients with fungal particles, the color of the particles was identical to the color of the fungal colonies that grew in the culture plate. Black particles associated with colonization by *Cladosporium* species were small and looked very similar to aseptic black particles.

Figure 2 — Microscopic analyses of aseptic particles shows (A1) crystal-like material (wet smear, 100× original magnification); (A2) an admixture of protein and cell debris (hematoxylin and eosin stain, 400× original magnification); (A3) crystal-like material [scanning electron microscopy (SEM), 3500× original magnification]; (B1,B2) fungal particles (wet smears, 100× and 400× original magnification respectively); and (B3) fungal particles (SEM, 750× original magnification).

The clinical course of the patients and the values of BG and GMI might be different depending on the fungal species (Table 2), but the data are too preliminary to draw conclusions. With respect to treatment, all patients with fungal particles received amphotericin B and underwent catheter removal. The colonized patients had grave outcomes, with almost of them transferring permanently to hemodialysis. One patient colonized with *Scedosporium* species died of sepsis (Table 2).

DISCUSSION

The presence of black particles in the PD system is not uncommon in Thai PD populations (2). Despite their uncertain cause, these particles seem to be harmless, especially in short-term follow-up; they can sometimes remain subclinical for several years. In contrast, fungal particles have been episodically reported in the literature (7–11), sometimes looking grossly similar to black particles, but with a seemingly paradoxical outcome. In most reported cases, the patient developed fungal peritonitis and technique failure. Notably, in

Figure 3 — (A) Levels of (1->3)-β-D-glucan and (B) the galactomannan index in peritoneal dialysis effluent from patients with aseptic black particles and fungal colonization at the time of Tenckhoff catheter removal. * $p < 0.0001$ between the groups.

TABLE 4
(1->3)-β-D-Glucan and Galactomannan Index in Peritoneal Effluent from Patients with Fungal Colonization When Fungal Particles Were First Observed in the Absence of Peritonitis

Pt ID	Sex	Age	Effluent cell count (cells/μL)	(1->3)-β-D-Glucan (pg/mL)	Galactomannan index	Particle color	Organism (identified by DNA sequencing)
1	Female	52	0	270	10.16	Black	<i>Alternaria alternata</i>
2	Male	67	2	132	1.24	Grey	<i>Cladosporium cladosporioides</i>
3	Male	72	8	230	6.12	Black	<i>Stachybotrys chartarum</i>

many of the patients, the presence of fungal particles preceded a full-blown fungal peritonitis (7–11). One patient had several recurrent fungal peritonitis episodes after longstanding fungal colonization (11). Close follow-up is necessary for the detection of fungal particles inside the TK catheter lumen. If particles are observed, early discrimination between fungal and aseptic black particles is challenging, because particles of both types can adhere tightly to the catheter wall and be hard to dislodge, causing difficulty in specimen collection. The present study demonstrates that effluent BG and GMI are interesting surrogate biomarkers for discriminating between the particle types.

Fungal peritonitis, an uncommon complication of PD treatment, is associated with high mortality and morbidity (4,23). More than half the patients with fungal peritonitis require a switch in dialysis modality because of alterations in peritoneal membrane function (23). In the present study, the duration on PD was shorter for patients with fungal particles than for patients with aseptic particles. The organisms most commonly isolated from fungal particles were molds that normally inhabit soil or water, indicating environmental contamination. *Cladosporium*, *Scedosporium*, and *Fusarium* are soil fungi (24–26). *Alternaria* is a common plant pathogen found in soil and water (27). *Simplicillium* is a freshwater fungus (28).

Our cases of fungal colonization emphasize the importance of training and retraining to reduce the risk of peritonitis as recommended by the International Society for Peritoneal Dialysis 2011 position statement for all PD patients (29). Notably, a causative organism of fungal peritonitis with dark particles in the present

study was a filamentous mold; in most published series, the common pathogens in fungal peritonitis have been *Candida* species (7). The explanation for our finding is not clear. Possible reasons include a more aggressive clinical course of candidiasis. Thus, a definite diagnosis of candidiasis can be made before visualization of *Candida* colonization. Or catheter composition might be more suitable for colony-building by filamentous mold. More study to clarify our observations are needed.

Most fungal colonizations were easily diagnosed by direct microscopic examination; however, obtaining specimens for both aseptic and fungal particles was very difficult because of the stickiness of particles. Aseptic particles were always black or brown; fungal particles could be gray, brown, black, or even white, depending on species. Fungal colonization should be highly suspected if the foreign particles are non-black in color, oval in shape, convex or cobblestone-like in appearance, skip-lesion-appearing, or very sticky, adhering to the catheter wall (Table 3, Figure 1). The color of the fungal particles in our study was consistent with the color of the associated cultured colony. More importantly, an early diagnosis of fungal colonization could prevent a subsequent episode of fungal peritonitis. Suspicion of fungal particles in a patient's catheter should prompt an active investigation for fungal infection and close follow-up.

Compared with culturing, a combination of BG and GMI assays might therefore be useful for the rapid diagnosis of a fungal colonization, guiding the physician to promptly manage potential infections (30–32). Compared with our patients having aseptic particles, all patients with fungal particles had very high BG and GMI levels (Figure 3). However, because of

Figure 4 — Cultured fungal colonies, with their associated direct KOH examination, from particles found inside the Tenckhoff catheter lumen (methylene blue stain to enhance fungi hyphae, 400× original magnification). (A1) *Alternaria* species. (A2) *Cladosporium* species. (A3) *Fusarium* species. (A4) *Simplicillium obclavatum*. (A5) *Scedosporium* species.

the low availability in the fungal cell wall of the components being tested for, BG detection of *Zygomycetes* and *Cryptococcus* is limited, as is GMI detection of *Candida* and *Acremonium* species (13,21,22). That BG limitation was not evident in the present study because no patient had a *Zygomycetes* infection. Concomitant use of both tests might be the better approach for discriminating fungal particles from aseptic black particles (19).

Some limitations of the present study have to be mentioned. First, the number of patients was small. Second, repeated measures to show the time-course of BG and GMI values were not available. The BG and GMI values when the fungal colonies first begin to present might be lower than diagnostic values. Third, only a small number of patients had fungal colonization with no fungal peritonitis. Most of the PDE was confounded by a fungal peritonitis. However, as a proof-of-concept study, our data are still valuable for demonstrating that BG and GMI in PDE are potential biomarkers for the presence of fungal colonies. Larger cohorts are needed to reach a more definitive conclusion.

CONCLUSIONS

Fungal colonization in the PD system is rare but serious complication for PD patients. Such colonies can sometimes be misperceived as the less-harmful manifestation of aseptic black

particles. Correct discrimination between these two conditions is essential for proper management. Direct microscopic examination of the particles is, in most cases, technically difficult because of their stickiness (adhering to the TK catheter), but we demonstrated that effluent BG and GMI are good surrogate biomarkers for a diagnosis of fungal colonization. Although dialysate GMI was relatively low in the presence of some fungal colonies, GMI might be helpful for detecting certain BG-negative fungal colonies. Manipulation of the catheter to dislodge dark particles should be avoided, and replacement of the TK catheter should be suspended until the cause of the particles is defined.

ACKNOWLEDGMENTS

The study was funded by Ratchadaphisek Sompoch Research Foundation, Faculty of Medicine, Chulalongkorn University and the National Center for Genetic Engineering and Biotechnology Research Fund (RES_55_122_30_016), Thailand National Science and Technology Development Agency. Fungitell kits were partially supported by Malcolm A. Finkelman, PhD, Director of Clinical Development, Associates of Cape Cod, MA, USA, with no relationship with the company. AL and KP are supported by a Grant for Development of New Faculty Staff, Chulalongkorn University. We also thank the staff of the PDICS Network, including King Chulalongkorn Memorial Hospital, Bangkok;

Sunpasitthiprasong Hospital, Ubon Ratchathani; Roi Et Hospital, Roi Et; Saraburi Hospital, Saraburi; Uttaradit Hospital, Uttaradit; and Buddhasothorn Hospital, Chachoengsao, for their endeavors and clinical pictures. Miss Uthaibhorn Singkham-in is thanked for her assistance.

DISCLOSURES

The authors have no financial conflicts of interest.

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