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Application of a Broad-Range Resequencing Array for Detection of Pathogens in Desert Dust Samples from Kuwait and Iraq▽

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A significant percentage of the human population is exposed to high levels of naturally occurring airborne dusts. Although the link between airborne particulate inhalation and a variety of respiratory diseases has long been established, little is known about the pathogenic role of the microbial component of the dust. In this study, we applied highly multiplexed PCR and a high-density resequencing microarray (RPM-TEI version 1.0) to screen samples of fine topsoil particles and airborne dust collected in 19 locations in Iraq and Kuwait for the presence of a broad range of human pathogens. The results indicated the presence of potential human pathogens, including *Mycobacterium*, *Brucella*, *Coxiella burnetii*, *Clostridium perfringens*, and *Bacillus*. The presence of *Coxiella burnetii*, a highly infectious potential biowarfare agent, was confirmed and detected in additional samples by use of a more sensitive technique (real-time PCR), indicating a high prevalence of this organism in the analyzed samples. The detection of potentially viable pathogens in breathable dusts from arid regions of Iraq and Kuwait underscores the importance of further study of these environments.

It is a well-established fact that the exposure of the human respiratory system to high concentrations of airborne particulate matter (dust) can have significant adverse health effects (15). Desert storm activity is the most significant source of nonoccupational dust exposure in arid and semiarid regions of the world. It is estimated that 0.5 to 5 billion tons of desert topsoil is made airborne every year during high-wind desert storm events globally (50). Exposure to desert dust has been linked to many conditions, including silicosis and asthma (9, 45, 48, 54). Arid areas of Iraq and Kuwait are among the largest sources of airborne dust on the Earth (61) due to the very high content of fine, easily suspendible particles in their topsoils (1). Many respiratory diseases that are linked or suspected to be linked with the inhalation of desert dust, such as desert lung syndrome (24, 25, 46, 51), desert storm pneumonitis (Al Eskan disease) (14, 31, 32), severe acute pneumonitis (12), and acute eosinophilic pneumonia (57), have been reported to occur in this region.

The pathogenic effect of dust inhalation on respiratory tissues is attributed mostly to direct physical action of the dust particles on the epithelium of the human airways and may be exacerbated by toxic effects of the trace elements (including arsenic, mercury, cadmium, and iron) and of biologically active compounds that the dust particles may carry (15). Not much is known, however, about the health impact of the microbiological component of the dust (bacteria, fungi, and viruses) on exposed populations. A few studies linked exposure to African desert dust with the increased risk of bacterial meningitis caused by *Neisseria meningitidis* in the "meningitis belt" of North Africa, but the role of the dust is not clear (8, 44, 58).

Previous research has shown that although desert dust particles are subjected to extreme physical conditions, such as UV irradiation, desiccation, lack of nutrients, and high temperatures, they contain surprisingly diverse viable microbes and bacterial and fungal spores (21, 28). Furthermore, while the mobilized desert topsoils are most often transported locally, large-scale high-energy wind events can result in long-range movement of the suspended particles, which in some cases cross oceans and even circumnavigate the globe (18, 19, 22). While some data indicate the possibility of intercontinental spread of infectious pathogens (17, 43, 56), very little is known about the role that long-distance airborne particulate transport plays in this phenomenon.

Screening dust for infectious agents requires a method that provides precise identification and a high sensitivity. A method having broad coverage would also be advantageous. The normal approaches, e.g., bacterial culture, microscopy, or molecular analysis of 16S and 18S rRNA, used to study the microbiological content of airborne desert dust do not meet all of these requirements (21, 27, 62). While microbiological culture allows for accurate identification of the bacteria found in the sample, it is capable of detecting only a small fraction of all of the microorganisms present, since <1% of the bacterial strains present in the environment are currently cultivable (3). Microscopic examination and 16S and 18S rRNA-based methods can give insight into the total biodiversity of the sample, but their resolution is limited to genus-level identification for many taxonomic groups (27, 62). Highly multiplexed detection technologies based on multiple molecular signatures with better overall resolution are necessary for screening environmental samples for human pathogens. High-density resequencing microarray technology, in particular, is an example of technology

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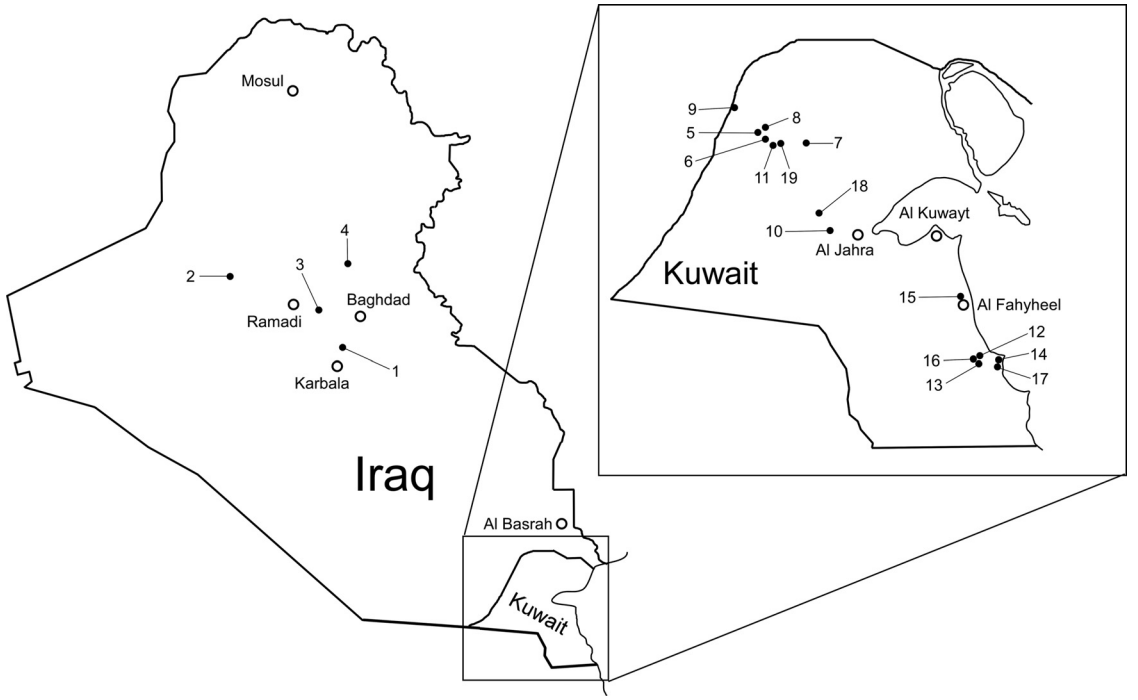

FIG. 1. Locations of sample collection sites. The sites are marked with filled circles and numbered. The open circles show the locations of major cities. All locations are approximate. The exact positions were recorded and are available upon request.

that was shown to be capable of highly accurate broad-range pathogen detection (6, 7, 36, 38, 59).

The focus of this study was to develop a procedure to screen dust samples for the presence of human pathogens that can provide immediate determination of their presence, which allows the directed use of more sensitive follow-on tests to confirm the presence of such pathogens. To achieve this goal, we used a high-density resequencing microarray designed to detect a broad range of biothreat agents, RPM-TEI version 1.0 (33), to analyze samples of the readily suspendible fraction of the desert topsoil and airborne particulates from 19 distinct locations in Kuwait and Iraq. In one case, detection of DNA of a known human pathogen with bioweapon potential (*Coxiella burnetii*) was investigated further using a highly sensitive real-time PCR assay.

MATERIALS AND METHODS

Soil and dust sample collection. (i) Time and location. Samples of soil and airborne particulates (dust) were collected between 23 July and 27 September 2007 from 4 locations in Iraq and 15 locations in Kuwait (Fig. 1 and Table 1), near the areas of deployment of U.S. troops, by the U.S. Navy Field Deployable Preventive Medicine Units (USN-FDPMUs).

(ii) Soil sample collection and processing. The center of the collection area was recorded by taking a global positioning system (GPS) reading (these data may be obtained directly upon request). Soil samples (to a maximum depth of 2 cm) were collected from the center of the collection area and from four additional positions (corresponding to the north, south, east, and west compass points) located 50 m from the center. Approximately 1 liter of surface soil was collected from each site (200 ml from each of the five positions). All soil samples were collected in sterile 50-ml tubes. Sterile working techniques were used for collection and all subsequent handling of the samples.

For each collection site, equal amounts of the soil from all five positions were mixed using sterile techniques to produce a composite sample representative of the area. The composite sample was subjected to a two-stage sieving process using sterilized standardized 3-in.-diameter stainless steel sieves (Gilson Company, Inc., Lewis Center, OH; Endecotts Ltd., London, England; and Arthur S. La Pine & Co., Chicago, IL). The sieves were used to sieve up to 50 g of soil, using 2 sieve stacks. Sieve stack 1 consisted of five sieves and a collection pan. The order of the sieves, from top (coarser) to bottom (finer), was sieve no. 20 (0.840-mm mesh), no. 40 (0.590-mm mesh), no. 60 (0.420-mm mesh), no. 80 (0.177-mm mesh), and no. 100 (0.149-mm mesh). The fraction of soil collected in the collection pan (particles of $<$0.149 mm) was applied to sieve stack 2, which consisted of four sieves and a collection pan. The order of the sieves from top to bottom was no. 140 (0.106-mm mesh), no. 200 (0.075-mm mesh), no. 270 (0.053-mm mesh), and no. 500 (0.025-mm mesh). The sieving was conducted using a Gilson performer III, model SS-3, sieve shaker (Gilson Company, Inc.) at an amplitude setting of 8 for 30 min. The soil fraction collected in the stack 2 collection pan, containing particles smaller than 0.025 mm (25 μm), was used for nucleic acid extraction. All sieves were cleaned using detergent solutions and sterilized by rinsing with 70% ethanol and irradiating with UV for 15 min before reuse.

(iii) Airborne particulate collection. Airborne particulate sampling at each collection site was performed using a deployable particulate sampler (DPS) system (SKC Inc., Eighty Four, PA) equipped with a PM10 size-selective inlet allowing collection of particles of 10 μm or smaller in aerodynamic diameter. The particles were collected for 24 h from the sampled air at a rate of 10 liters per min on 47-mm, 2-μm-pore-size polytetrafluoroethylene (PTFE) filters reinforced with a PMP support ring (SKC Inc.). In each location, a duplicate PTFE filter was taken to the field but not exposed and was used as a negative control (field blank [FB]). The exposed filters were stored short term (approximately 1 month), shipped to the laboratory at ambient temperatures (approximately 25°C), and frozen at −20°C for long-term storage upon arrival at the laboratory. The blank filters were weighed before the exposure to airborne particulates, and filters containing collected particulates were weighed after arriving back at the laboratory. The weight of collected particulates was obtained by subtracting the weight of the empty filter from the combined weight of the filter with particulates (Table 2).

Nucleic acid extraction. The total nucleic acids were extracted from filter-captured airborne particulates and topsoil samples by use of a FastDNA spin kit for soil (MP Biomedicals, Solon, OH) according to the manufacturer's instructions. For the extraction of nucleic acids from soil samples, 500 mg of the sub-25-μm fraction of composite soil samples was used. The final nucleic acid elution was performed using 50 μl of DNase- and pyrogen-free water. In order to extract nucleic acids from airborne dust particles collected on PTFE filters, the

TABLE 1. Collection site information

Site no.	Country	Location	Site[a]	Type[b]	Environmental conditions[c]		
					Temp (°C)	Humidity (%)	Wind speed (m/s)
1	Iraq	Iskandariyah FPB	Transformer yard	SA/H	36.9	25.4	1.2
2		Al-Asad airbase	South of flight line	A/H	35.6	12.5	5.0
3		Camp Falluja	Open field	SA/M	33.7	23.9	2.6
4		Camp Anaconda	Near firing range	SA/M	34.1	18.1	3.3
5	Northern Kuwait	Range 6	Open field	A/M	36.0	11.0	3.3
6		Range 7A	Open field	A/M	29.2	15.4	0.8
7		Camp Buehring	Staging area	A/M	54.4	6.0	4.4
8		FOB Scimitar	Open field	A/M	36.0	11.0	3.3
9		K-Crossing	Open field	SA/M	41.9	13.3	1.8
10		Life support area	Open field	SA/M	42.3	9.8	5.4
11		Range 8 ECP	Open desert	A/M	31.9	6.0	2.5
12	Southern Kuwait	Camp Arifjan	Near tent area	E/M	36.1	30.0	2.2
13		Camp Arifjan	Open field	A/ND[d]	42.8	7.3	0.0
14		Camp Patriot	Commando cell KNB	A/M	35.8	26.5	1.3
15		SPOD	Eastern connex yard	E/M	38.9	12.1	2.8
16		Camp Arifjan	Zone 6, near ballfield	A/M	37.1	13.0	5.8
17		Camp Patriot	Berthing spaud	A/H	36.8	54.0	2.2
18	Northern Kuwait	Camp Virginia	Open field	A/M	40.4	10.0	3.7
19		FOB Lance	Open desert	E/L	37.6	20.7	8.0

[a] The approximate geographic locations of collection sites within Iraq and Kuwait are depicted in Fig. 1. The exact GPS coordinates of the sites were collected and are available upon request.

[b] The type of location is defined by the type of vegetation present (code before the slash) and the degree of land transformation (code after the slash). Vegetation codes: SA, semiarid—desert scrublands and grasses; A, arid—diffuse, sparse vegetation; E, extremely arid—no vegetation. Land transformation codes: H, high—farm use, camp use, heavy traffic; M, medium—lightly populated, light traffic; L, low—remote, at least 8 km from populated areas, no traffic.

[c] Environmental condition readings were taken at the time of topsoil collection, which coincided with the starting point of airborne particulate collection, which lasted 24 h.

[d] Degree of land transformation was not recorded for this location. ND, no data.

PMP support ring was removed and the whole filter was used for extraction. The filters were folded and inserted into the kit's lysing matrix E tube and subsequently processed in the same way as soil samples. The field blanks were extracted in the same way as the exposed filters. The final nucleic acid elution for airborne particulate samples was performed using 100 μl of DNase- and pyrogen-free water. The DNA concentrations were measured fluorometrically using a Qubit instrument and Qubit dsDNA HS and BR assay reagent kits (Invitrogen, Carlsbad, CA).

TABLE 2. Amounts of airborne particulates collected and DNA contents of collected samples[a]

Site no.	Airborne particulates			Topsoil DNA content[b] (μg/g)
	Total collected (mg)	Total DNA (ng)	DNA content (μg/g)	
1	1.9	22.8	12.0	20.8
2	1.3	37.4	28.2	15.3
3	0.6	17.6	28.2	10.8
4	2.2	16.4	7.5	13.6
5	1.4	ND	ND	9.2
6	1.5	ND	ND	6.0
7	1.9	ND	ND	3.6
8	1.6	16.4	10.1	8.6
9	2.4	12.4	5.2	16.4
10	1.6	24.0	15.0	6.8
11	2.6	ND	ND	9.8
12	4.2	ND	ND	2.0
13	ND	ND	ND	7.7
14	2.1	12.5	5.9	1.0
15	2.4	13.8	5.7	5.2
16	4.4	ND	ND	2.0
17	2.7	ND	ND	9.7
18	1.8	22.8	12.6	3.3
19	3.6	46.1	13.0	13.0

[a] Data were not obtained for some of the airborne particulate samples. ND, no data.

[b] The topsoil DNA content is based on total DNA extracted from 500 mg of topsoil fraction containing particles smaller than 0.025 mm.

RPM-TEI version 1.0 microarray analysis. The RPM-TEI version 1.0 (subsequently called RPM-TEI) microarray analysis was conducted as previously described (33) and as summarized in Fig. 2. Briefly, 2 μl of nucleic acid preparation from each soil sample, airborne particulate sample, and field blank was reverse transcribed using random primers to obtain cDNAs for RNA templates potentially present in the sample. The obtained mixture was split into four aliquots of equal volume, and four separate, approximately 50-plex PCRs were conducted using RPM-TEI microarray-specific primer cocktails in order to amplify all of the targets (see reference 33 for detailed information on multiplex PCR mixtures). Subsequently, the PCR amplification products were combined and purified using a QIAquick PCR purification kit (Qiagen, Valencia, CA). Purified PCR products were fragmented using DNase I and labeled with biotinylated ddATP, using terminal transferase. Labeled samples were loaded into preheated RPM-TEI microarray cartridges (TessArae LLC, Potomac Falls, VA) that were previously incubated with prehybridization buffer for at least 10 min, and the hybridization was conducted overnight at 49°C. Next, the sample was removed from the cartridge and the microarray was processed using a GeneChip 450 fluidics station and scanned using a GeneChip 3000 scanner with an autoloader (Affymetrix, Santa Clara, CA). Sequence data based on microarray hybridization patterns were generated by GeneChip 4.0 sequence analysis software (Affymetrix). Pathogen identification was performed by using Computer-Implemented Biological Sequence Identifier (CIBSI) 2.0 software (40), which filters the sequence of base calls from the microarray, and then using the resolved bases as the query for a similarity search of a DNA database (currently NCBI GenBank) to identify the most likely species and variants that matched the base calls from the hybridization observed. Although the microarray was designed with tiles for specific pathogen targets (33), it is capable of detection and correct sequence determination for targets differing by up to 15% from the sequence present on the microarray. This allows for detection of target variants and near-neighbor discrimination. For RPM-TEI, one to six different genome/plasmid sequences per targeted pathogen are present on the microarray. Depending on the concentration and quality of the target, similarity to targeted pathogen, and other factors, the hybridization on the microarray may produce anywhere from nearly complete resequencing of the detected target to short gene sequence fragments to no signal at all. Based on a similarity search of the obtained sequences with GenBank, CIBSI software will provide one of the following four outcomes: (i) no

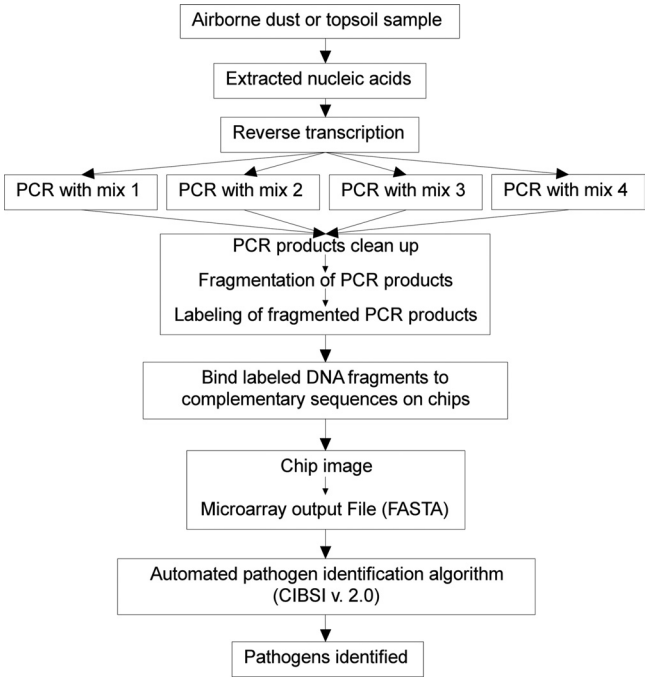

FIG. 2. Overview of the experimental protocol used with the RPM-TEI version 1.0 microarray. (Adapted from reference 37.)

target was detected, (ii) a targeted pathogen was detected, (iii) a near neighbor was detected, and (iv) either a targeted pathogen or a near neighbor was detected, but the microarray sequence data were insufficient to discriminate between them.

Real-time PCR. Real-time PCR for detection of *Coxiella burnetii* (IS*1111* marker) was performed using previously published PCR primers IS1111-F801 (5′-AATTTCATCGTTCCCGGCAG-3′) and IS1111-R901 (5′-GCCGCGTTTA CTAATCCCCA-3′) (13). The amplification reactions were conducted using SsoFast EvaGreen reaction mix (Bio-Rad Laboratories, Hercules, CA) according to the manufacturer's instructions. An aliquot of 2 μl of total nucleic acid preparation from each soil, airborne particulate, or field blank sample was used as a template. The reaction was carried out in a 20-μl total volume containing a 500 nM concentration of each primer. Thermal cycling and fluorescent signal detection were performed in a CFX96 real-time PCR detection system (Bio-Rad Laboratories) with the following thermal cycling conditions: initial incubation at 98°C for 2 min, followed by 40 cycles of 98°C for 2 s and 60°C for 5 s. The amplification cycle was followed by melting curve analysis under the following conditions: samples were heated from 75°C to 95°C with 0.2°C increments, and the fluorescence was read after 10 s of incubation at each step. The results were analyzed with CFX Manager v. 1.5.534.0511 software (Bio-Rad Laboratories). A 5-μl aliquot of each *C. burnetii*-positive sample was analyzed by agarose gel electrophoresis (1.2% FlashGel DNA cassette; Lonza, Walkersville, MD) to confirm that the obtained PCR products had the expected size.

RESULTS

RPM-TEI microarray analysis. Nucleic acids extracted from all samples (soil, airborne particulate, and field blank samples) were analyzed using a high-density resequencing microarray for detection of tropical and emerging pathogens (RPM-TEI). Microarray analysis indicated that several microorganisms were present in the analyzed samples. The most common bacteria present in these samples were *Mycobacterium* spp., followed by *Brucella* spp. and *Coxiella burnetii. Clostridium perfringens* was found in 3 samples, while *Bacillus* spp. were found at only one site, in Iraq. The results are summarized in Table 3.

Almost all soil samples and the majority of airborne particulate samples (with the exception of a soil sample from site 14 and airborne particulate samples from sites 6, 8, 13, and 17) were found to contain DNA that hybridized to microarray tiles designed for detection of the *rpoB* gene of *Mycobacterium tuberculosis*, but other sequences of *M. tuberculosis* were not positive. The possibility of cross contamination was excluded by inclusion and analysis of negative controls (field blanks), all of which were negative for the presence of *Mycobacterium*. The sequences read by the microarray indicated, however, that the detected DNAs did not belong to *M. tuberculosis*. They were most similar to sequences of various *Mycobacterium* species belonging to the group of rapidly growing mycobacteria (RGM). These species (depending on the particular sample) included *Mycobacterium barrassiae, Mycobacterium gilvum, Mycobacterium smegmatis*, and *Mycobacterium ulcerans*. In some cases, determination of a single most likely species was impossible due to insufficient sequence data.

A large number of analyzed samples contained nucleic acids hybridizing with the *gyrA* tile for *Brucella melitensis*. Approximately half of the soil samples (from sites 5, 6, 9, 10, 12, 13, 15, and 19) and one airborne particulate sample (from site 1) contained detectable quantities of this DNA. The sequences obtained were found to be most similar to those of *Brucella suis, Brucella abortus*, or *B. melitensis*, but the sequence information was not sufficient to make a definitive determination of which of these three species was present in the sample. In one case (soil from site 18), the sequences obtained from this tile were determined to be most similar to GenBank sequences of *Bradyrhizobium*, although species-level determination was impossible.

The presence of *C. burnetii* nucleic acids was detected in four samples (soil from sites 5 and 8 in Northern Kuwait and airborne particulates from locations 12 and 16 in Southern Kuwait). In all four cases, strong hybridization with the *Coxiella* IS*1111* tile was observed. The quality of the sequences obtained from this tile allowed for species-level identification. With the airborne particulate sample from site 16, hybridization with microarray tiles containing an additional *Coxiella* diagnostic sequence, *icd*, was also detected.

Nucleic acid sequences of another human pathogen, *Clostridium perfringens*, were found in 3 samples (soil from location 17 and airborne particulates from sites 3 and 16). The species-level identifications in the case of this organism were made based on hybridization with *gyrA* tiles for samples from locations 3 and 16 and with the *colA* tile for the airborne particulate sample from site 17.

Sequences hybridizing with *Bacillus rpoB* tiles were found in both sand and airborne particulates collected in Iraq (site 1). The obtained sequences allowed for determination that detected nucleic acids originated from *Bacillus* bacteria belonging to the *Bacillus cereus* group but excluded the possibility that they were from any *Bacillus anthracis* strain with a sequence submitted to GenBank.

Real-time PCR detection of *Coxiella burnetii* DNA. The detection in these samples of *Coxiella burnetii*, the etiological agent of Q fever and a potential bioweapon, by use of RPM-TEI raised interest and concern that warranted further investigation. Real-time PCR using primers designed by Christensen et al. (13) was conducted to confirm the findings of the

TABLE 3. RPM-TEI v. 1.0 microarray identification results[a]

Country	Site no.	Sample type[c]	Hybridizing detector tile		Most likely identification[d]
			Organism	Gene or sequence	
Iraq	1	S	*B. anthracis*	*rpoB*	*Bacillus cereus* group
		S	*M. tuberculosis*	*rpoB*	*Mycobacterium barrassiae*
		A	*B. anthracis*	*rpoB*	*Bacillus cereus* group
		A	*Brucella melitensis*	*gyrA*	*Brucella suis, B. abortus*, or *B. melitensis*
		A	*M. tuberculosis*	*rpoB*	*Mycobacterium barrassiae*
	2	S	*M. tuberculosis*	*rpoB*	*Mycobacterium barrassiae*
		A	*M. tuberculosis*	*rpoB*	*Mycobacterium gilvum*
	3	S	*M. tuberculosis*	*rpoB*	*Mycobacterium barrassiae*
		A	*Clostridium perfringens*	*gyrA*	*Clostridium perfringens*
		A	*M. tuberculosis*	*rpoB*	*Mycobacterium barrassiae*
	4	S	*M. tuberculosis*	*rpoB*	*Mycobacterium barrassiae*
		A	*M. tuberculosis*	*rpoB*	*Mycobacterium gilvum* or *M. smegmatis*
Northern Kuwait	5[b]	S	*Brucella melitensis*	*gyrA*	*Brucella suis, B. abortus*, or *B. melitensis*
		S	*Coxiella burnetii*	IS*1111*	*Coxiella burnetii*
		S	*M. tuberculosis*	*rpoB*	*Mycobacterium barrassiae*
	6	S	*Brucella melitensis*	*gyrA*	*Brucella suis, B. abortus*, or *B. melitensis*
		S	*M. tuberculosis*	*rpoB*	*Mycobacterium barrassiae*
		A	No hybridization		
	7	S	*M. tuberculosis*	*rpoB*	*Mycobacterium barrassiae*
		A	*M. tuberculosis*	*rpoB*	*Mycobacterium smegmatis*
	8	S	*Coxiella burnetii*	IS*1111*	*Coxiella burnetii*
		S	*M. tuberculosis*	*rpoB*	*Mycobacterium gilvum* or *M. barrassiae*
		A	No hybridization		
	9	S	*Brucella melitensis*	*gyrA*	*Brucella suis, B. abortus*, or *B. melitensis*
		S	*M. tuberculosis*	*rpoB*	*Mycobacterium barrassiae*
		A	*M. tuberculosis*	*rpoB*	*Mycobacterium gilvum, M. smegmatis, M. barrassiae*, or *M. vanbaalenii*
	10	S	*Brucella melitensis*	*gyrA*	*Brucella abortus* or *B. melitensis*
		S	*M. tuberculosis*	*rpoB*	*Mycobacterium barrassiae*
		A	*M. tuberculosis*	*rpoB*	*Mycobacterium gilvum, M. vanbaalenii*, or *M. lepromatosis*
	11	S	*M. tuberculosis*	*rpoB*	*Mycobacterium barrassiae*
		A	*M. tuberculosis*	*rpoB*	*Mycobacterium gilvum* or *M. vanbaalenii*
Southern Kuwait	12	S	*Brucella melitensis*	*gyrA*	*Brucella abortus* or *B. melitensis*
		S	*M. tuberculosis*	*rpoB*	*Mycobacterium barrassiae*
		A	*Coxiella burnetii*	IS*1111*	*Coxiella burnetii*
		A	*M. tuberculosis*	*rpoB*	*Mycobacterium barrassiae* or *M. mageritense*
	13	S	*Brucella melitensis*	*gyrA*	*Brucella abortus* or *B. melitensis*
		S	*M. tuberculosis*	*rpoB*	*Mycobacterium barrassiae*
		A	No hybridization		
	14	S	No hybridization		
		A	*M. tuberculosis*	*rpoB*	*Mycobacterium barrassiae* or *M. fortuitum*
	15	S	*Brucella melitensis*	*gyrA*	*Brucella suis* or *B. melitensis*
		S	*M. tuberculosis*	*rpoB*	*Mycobacterium barrassiae*
		A	*M. tuberculosis*	*rpoB*	*Mycobacterium barrassiae*
	16	S	*M. tuberculosis*	*rpoB*	*Mycobacterium barrassiae*
		A	*Clostridium perfringens*	*gyrA*	*Clostridium perfringens*
		A	*Coxiella burnetii*	*icd*	*Coxiella burnetii*
		A	*Coxiella burnetii*	IS*1111*	*Coxiella burnetii*
		A	*M. tuberculosis*	*rpoB*	*Mycobacterium barrassiae*
	17	S	*Clostridium perfringens*	*colA* (kappa toxin)	*Clostridium perfringens*
		S	*M. tuberculosis*	*rpoB*	*Mycobacterium ulcerans*
		A	No hybridization		
Northern Kuwait	18	S	*Brucella melitensis*	*gyrA*	*Bradyrhizobium*
		S	*M. tuberculosis*	*rpoB*	*Mycobacterium barrassiae*
		A	*M. tuberculosis*	*rpoB*	*Mycobacterium barrassiae*
	19	S	*Brucella melitensis*	*gyrA*	*Brucella suis, B. abortus, B. ovis*, or *B. melitensis*
		S	*M. tuberculosis*	*rpoB*	*Mycobacterium ulcerans*
		A	*M. tuberculosis*	*rpoB*	*Mycobacterium barrassiae*

[a] All field blanks (FB samples) were negative for all microarray tiles.
[b] No airborne particulate was collected for this location.
[c] S, soil; A, airborne particulate.
[d] "Most likely identification" was a determination made by CIBSI 2.0 software based on matching sequences obtained from the RPM-TEI microarray with sequences in GenBank. See Materials and Methods and reference 35 for details.

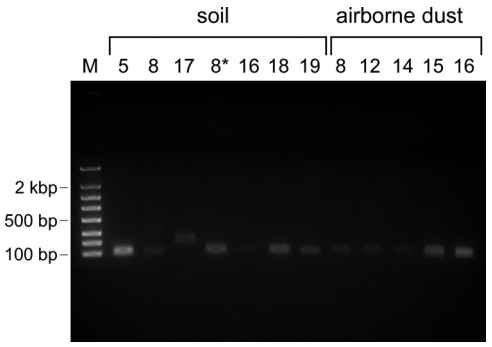

FIG. 3. Amplification products of real-time PCRs for *Coxiella burnetii* detection. Lane M, FlashGel DNA marker (100 bp to 4 kb). Remaining lanes are labeled with the site number where the sand or airborne particulate was collected. The product of expected size (101 bp) was amplified in all cases except for sample 17, which was determined to be negative based on amplicon sequencing (data not shown). The real-time PCR using the soil sample from location 8 was run in duplicate, using two independently extracted DNA preparations (soil lanes 8 and 8*).

microarray and to possibly detect the presence of this organism in additional samples. Similar to the RPM-TEI microarray, this assay takes advantage of the multiple copies of the IS*1111* marker present in the *C. burnetii* genome (30), and IS*1111* was previously shown to be very specific and sensitive (13, 26, 39) for *C. burnetii* detection.

The *C. burnetii*-specific real-time PCR was run on all collected samples. The field blanks and water were included as negative controls. The PCR analysis confirmed the presence of *Coxiella*-specific nucleic acids in all samples that tested positive for *Coxiella* by the RPM-TEI microarray (soil from sites 5 and 8 and airborne dust from sites 12 and 16) but also detected the presence of *C. burnetii* DNA in six additional samples: soil from sites 16, 18, and 19 and airborne particles from sites 8, 14, and 15. Agarose gel electrophoresis of the amplification products confirmed their correct sizes (Fig. 3). One additional sample (soil from site 17) was found to give a positive amplification; however, analysis of the amplification product (agarose gel electrophoresis, melting curve analysis, and sequencing) indicated that this was a false-positive result. In total, *C. burnetii* was detected (in either soil or dust) in almost half (8 of 19) of all tested locations. All field blank samples and water resulted in no amplification.

DISCUSSION

In this study, we investigated the potential for sand and airborne dust to contain known human pathogens in samples collected from a number of locations in Iraq and Kuwait. Analysis conducted using an RPM-TEI microarray allowed for detection of nucleic acids of a number of well-known human pathogens, such as *Coxiella burnetii* and *Clostridium perfringens*, which may cause disease by inhalation. Nucleic acids were also detected for genera such as *Mycobacterium, Bacillus*, and *Brucella*, important potential human pathogens that may also be spread by the respiratory route. Some of them (*Coxiella* and *Brucella*) require specialized culture conditions that are not available in most diagnostic laboratories and would not be detected by plating on blood agar, and therefore molecular techniques facilitate their detection. While it was not possible to determine if the detected pathogens were viable based on the microarray results, the detection of their nucleic acids warrants further studies on the health risk associated with breathing fine particles from arid, dusty regions of the Middle East. It should also be noted that there was no significant correlation between the environmental conditions at the collection site and the pathogens detected (Table 1). The analysis also showed that the DNA contents in both airborne dust samples and topsoil were generally within 1 order of magnitude (from micrograms to tens of micrograms per gram of sample) and failed to show any significant connection between the amount of the airborne particulate collected or overall sample DNA content and the pathogen nucleic acids detected (Table 2).

The two most prevalent bacterial targets found that can act as human pathogens were *Mycobacterium* spp. (11, 60) and *Brucella* spp. The mycobacteria are commonly found in soil (35, 47), and some RGM might be involved in respiratory disease in humans (2, 49). *Brucella* spp. include major zoonotic pathogens causing brucellosis in livestock. In addition, some *Brucella* species (e.g., *B. suis* in the United States) have been weaponized in the course of offensive biological weapon programs (20). Human infections with *Brucella* species (mostly with *B. melitensis* and *B. abortus*) usually occur from direct or indirect exposure to infected animals (63), but direct infection via inhalation of pathogen-contaminated aerosols is possible (63).

A more serious potential threat to humans detected in a number of analyzed samples was *Coxiella burnetii*, which causes Q fever and is known to be able to persist for long periods in the environment (41, 52). The identification of this organism by RPM-TEI microarray analysis was based mostly on detection of the IS*1111* insertion sequence, which is specific to this species and present in its genome in multiple copies (ranging from 7 to 110) (30). The natural amplification of IS*1111* may explain the fact that this marker was detected in all four *C. burnetii*-positive samples, while the *icd* marker was detected in only one. A sensitive real-time PCR test confirmed the microarray positive results and revealed the presence of *C. burnetii* DNA in additional locations, indicating a significant prevalence of this organism in the desert dust in Kuwait. The fact that Q fever was previously observed in this region and the several cases reported for U.S. troops deployed to this part of the world (4, 5, 16, 23, 34) are consistent with our findings. A recent large survey of environmental samples collected from six geographically distinct parts of the United States found that 23.8% of all analyzed samples were *Coxiella* positive by real-time PCR, suggesting the ubiquity of *C. burnetii* in the environment, which raises the interesting question of how closely related the organisms are and whether they may be transported intercontinentally or globally in dust particles (29).

Finding nucleic acids for organisms belonging to the genus *Bacillus* in samples from only one location was surprising, since *Bacillus* is relatively common in soil. This might have been the result of marker selection geared toward *Bacillus anthracis* detection (there are 6 markers specific for *B. anthracis*, including 2 chromosomal and 4 plasmid-borne markers, and only 1

for *B. cereus* on the microarray). Another possibility is poor nucleic acid recovery from *Bacillus* spores.

It is worth nothing that all microorganisms for which nucleic acids were detected by microarray analysis, with the possible exception of *Mycobacterium*, are predominantly zoonotic in nature. This could be the result of sampling in the vicinity of agricultural areas, where infected animals (cattle, sheep, and goats) are likely to contaminate the soil. Also, no viral pathogens were detected, even though the RPM-TEI array is capable of detecting a wide array of viruses. This could be the result of the genuine absence of viral particles from the dust particles due to very harsh environmental conditions affecting the desert dust or an experimental bias introduced by the nucleic acid extraction methods. It should also be noted that although fungi and/or fungal spores may have significant impacts on human health (53, 55), the RPM-TEI array was not designed to detect fungal pathogens. Future development will include potential human fungal pathogens in the microarray design to expand the coverage to this class of pathogens.

In summary, the results of this study indicate that high-density resequencing microarrays have great potential as a tool for broad-spectrum screening of environmental samples for the presence of human pathogens. The RPM-TEI microarray directly detected potential human pathogens and also guided the use of more specific and sensitive follow-up tests. One of the main strengths of the RPM technology, as shown by this study, lies in its ability for simultaneous detection of hundreds of pathogens in a single test. Other sensitive molecular methods, such as real-time PCR, are usually characterized by low multiplexing levels. While these assays are cheaper as standalone tests, running enough of them to provide the same coverage of pathogens with a single sample quickly overtakes RPM microarrays in both cost and logistical complexity of running the analysis. Although other technologies with universal coverage that may be applied to analyze environmental samples exist, they either have low resolution (such as 16S rRNA-based techniques) or are much more complex and expensive (such as high-throughput *de novo* sequencing).

This study also gives a glimpse at the baseline levels of recognized biothreat agents and their near neighbors that are naturally present in the environment. A number of studies aiming to determine natural background levels have been conducted (10, 42); however, more are needed in order to develop better environmental biothreat sensing technologies and algorithms that are significantly less prone to false-positive results than currently available systems.

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