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Absence of *Mycobacterium intracellulare* and Presence of *Mycobacterium chimaera* in Household Water and Biofilm Samples of Patients in the United States with *Mycobacterium avium* Complex Respiratory Disease

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Recent studies have shown that respiratory isolates from pulmonary disease patients and household water/biofilm isolates of *Mycobacterium avium* could be matched by DNA fingerprinting. To determine if this is true for *Mycobacterium intracellulare*, household water sources for 36 patients with *Mycobacterium avium* complex (MAC) lung disease were evaluated. MAC household water isolates from three published studies that included 37 additional MAC respiratory disease patients were also evaluated. Species identification was done initially using nonsequencing methods with confirmation by internal transcribed spacer (ITS) and/or partial 16S rRNA gene sequencing. *M. intracellulare* was identified by nonsequencing methods in 54 respiratory cultures and 41 household water/biofilm samples. By ITS sequencing, 49 (90.7%) respiratory isolates were *M. intracellulare* and 4 (7.4%) were *Mycobacterium chimaera*. In contrast, 30 (73%) household water samples were *M. chimaera*, 8 (20%) were other MAC X species (i.e., isolates positive with a MAC probe but negative with species-specific *M. avium* and *M. intracellulare* probes), and 3 (7%) were *M. avium*; none were *M. intracellulare*. In comparison, *M. avium* was recovered from 141 water/biofilm samples. These results indicate that *M. intracellulare* lung disease in the United States is acquired from environmental sources other than household water. Nonsequencing methods for identification of nontuberculous mycobacteria (including those of the MAC) might fail to distinguish closely related species (such as *M. intracellulare* and *M. chimaera*). This is the first report of *M. chimaera* recovery from household water. The study underscores the importance of taxonomy and distinguishing the many species and subspecies of the MAC.

Previous studies have suggested household water (especially from bathroom showers) as a source of the *Mycobacterium avium* complex (MAC), which causes chronic lung disease (1, 2, 3, 4, 5). Both *M. avium* and *M. intracellulare* have been recovered from sputum, sinus, and household or potable water samples in multiple countries, including the United States and Japan (1, 4–8). In the majority of these studies, either hybridization probe methods (AccuProbe; Hologic Gen-Probe, Inc., San Diego, CA) or a multiplex 16S rRNA gene PCR was used for identification (1, 5, 9, 10). Isolates positive with the MAC probe but negative with the species-specific *M. avium* and *M. intracellulare* probes are collectively referred to as MAC X species.

Currently, the *M. avium* complex includes four *M. avium* subspecies (*M. avium* subsp. *avium*, *M. avium* subsp. *hominissuis*, *M. avium* subsp. *silvaticum*, and *M. avium* subsp. *paratuberculosis*) (11, 12) and eight species (*M. avium*, *M. intracellulare*, *M. marseillense* [13], *M. timonense* [13], *M. bouchedurhonense* [13], *M. colombiense* [14], *M. vulneris* [15], and *M. chimaera* [16]). The last of these species (*M. chimaera*) was first reported in 2004 by Tortoli et al., who described 12 isolates of a slowly growing species closely related to *M. intracellulare* and recovered from respiratory samples in Italy, including some from patients with definite lung infection (16). The new species differed by only 1 bp at position 450 in the entire 16S rRNA gene sequence of *M. intracellulare* but had a very different 16S to 23S internal transcribed spacer (ITS) region sequence (16). Tortoli and coauthors named this new species *M. chimaera*, as it included characteristics of several species and masqueraded as *M. intracellulare* (16).

We recently completed a study of variable-number tandem-repeat (VNTR) typing of clinical isolates of *M. intracellulare* using ITS sequencing to confirm the species designation (7). We then began household water biofilm studies for some of our patients and performed species identification and VNTR genotyping for MAC isolates as needed.

Preliminary studies suggested that respiratory isolates of *M. intracellulare* indeed belonged to that species based on ITS sequencing (7) but that biofilm isolates thought to be *M. intracellulare* were actually *M. chimaera* (R. J. Wallace, Jr., unpublished observation). We then expanded the household study to reassess water and biofilm isolates from three additional published studies

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TABLE 1 Demographics of U.S. household water studies for the MAC

Study no.	Type of disease	No. of patients	No. of households	No. of culture sites	Culture source		Location(s)	Reference(s)
					Water	Biofilm		
1	Sinus	8	8	80	+	+	New York City, San Francisco, CA, and Florida	6
2	Pulmonary	26	26	224	−	+	Philadelphia, PA	L. Lande, unpublished data; 17
3	Pulmonary	28	30	379	+	+	14 states	1
4	Pulmonary	10	15	66	−	+	Texas and Louisiana	R. J. Wallace, Jr., unpublished data
5	Pulmonary	1	1	3	+	+	New York City	2
Total		73	80	752				

(1, 2, 6) and one additional unpublished respiratory study in an attempt to validate this initial finding (17).

MATERIALS AND METHODS

Patients and isolates. Patients with MAC nodular lung disease who were part of long-term studies at the University of Texas Health Science Center at Tyler (UTHSCT) and who had been diagnosed with a recent episode of active MAC lung disease in the past 6 months were recruited for household water biofilm studies. Sputum isolates of the MAC recovered from these patients during the previous 5 years were available. The patient and household water biofilm studies were done using a protocol approved by the human subjects committee of the UTHSCT.

Environmental household water and biofilm isolates identified as *M. intracellulare* were also obtained from one previously published study of mycobacterial sinusitis (6) and two previously published studies of pulmonary disease (1, 2), as well as an additional unpublished study of pulmonary disease comparing household biofilm and clinical MAC isolates (17) (Table 1). Cultures for each of these four studies were prepared in the same laboratory (Virginia Polytechnic Institute and University, Blacksburg, VA) with recovered MAC isolates that had been frozen until the time of the current study. Each study was approved by the human subjects committee for the involved institution.

Control strains for PCR and sequencing included *M. intracellulare* strain ATCC 13950^T and *M. chimaera* strain DSM 44623^T.

Household water sampling. The five studies utilized water samples and/or swab cultures of bathroom and kitchen faucet filters and pipes and showerhead filters and showerhead pipes, as well as samples from any other potential sites (e.g., air filters, hot tub filters, and bathtub inlet pipes, etc., when available) (Table 1). Water samples of 500 ml were concentrated by centrifugation (500 × *g* for 20 min), and then the pellet was resuspended in 1 ml of sterile tap water. Swabs were added to 2 ml of sterile tap water. Samples were decontaminated using the method of Thomson et al. (18) and spread onto Middlebrook 7H10 agar plates that were incubated for 3 weeks and then screened for acid-fast bacilli (AFB) (1, 2, 19).

Nonsequencing identification of *M. intracellulare* and *M. avium*. Sputum isolates were identified as members of the MAC for all studies using a commercial DNA probe (AccuProbe). Sputum or water/biofilm isolates of MAC were initially identified as *M. avium* or *M. intracellulare* by nonsequencing methods using an *M. avium* or *M. intracellulare* DNA probe (AccuProbe) (10), a multiplex 16S rRNA gene PCR (9), or an *hsp65* PCR of a 441-bp sequence followed by digestion with BstEII or HaeIII as described by Telenti et al. (20).

DNA sequencing for species identification of *M. intracellulare* and *M. avium*. Sequencing of the 280-bp 16S to 23S internal transcribed spacer (ITS) region of each isolate of *M. intracellulare* (based on the preliminary identification) and the two reference strains was performed as described previously (11, 12, 16, 21). This method readily distinguishes the usual *M. intracellulare* sequence (Min-A) (7, 21) from the *M. chimaera* sequence MAC-A (16, 21). Sequences were compared to the Min-A sequence for *M. intracellulare* type strain ATCC 13950^T (GenBank sequence no. AB026691) and the MAC-A sequence for *M. chimaera* type strain DSM 44623^T (GenBank sequence no. EF521902).

Partial 16S rRNA gene sequencing of the first 500 bp of isolates identified as *M. chimaera* by the ITS region was also performed (7). This sequence contains the single base pair difference between *M. intracellulare* and *M. chimaera* (16).

Thirty-five *M. avium* isolates identified by nonsequencing methods also underwent ITS region sequencing. No nontuberculous mycobacteria (NTM) species other than *M. avium* was identified, which demonstrates that species confusion similar to that seen with *M. intracellulare* and *M. chimaera* is not seen with *M. avium*.

RESULTS

Patients. A total of 73 patients with MAC respiratory disease were studied (Table 1); 65 met the American Thoracic Society (ATS) criteria for MAC lung disease (22). Isolates from eight patients with mycobacterial sinusitis and from their household water were also included for study (6). The patients were from multiple states and two large cities in the United States (Table 1).

Isolates and nonsequencing species identification. One or more sputum culture isolates were available for 53 of the patients with MAC lung disease. Multiple sputum isolates were available from the 10 UTHSCT patients, many of whom had had prior episodes of MAC lung disease caused by species other than *M. intracellulare* or had mixed infections at the time of the water studies (study group 4; Tables 1 to 3). For the other lung disease patients, only a single respiratory culture was available (study groups 1 to 3 and 5; Tables 1 to 3), as was also the case for the patients with sinusitis.

A comparison of the results from the commercial DNA *M. intracellulare* probe (AccuProbe) for isolates of *M. intracellulare* and *M. chimaera* showed that both species gave strongly positive results. A comparison of these two species using the multiplex 16S rRNA gene PCR (9) is shown in Fig. 1, and a comparison of the *hsp65* PCR restriction enzyme analysis (PRA) (20) is shown in Fig. 2. None of these three nonsequencing methods distinguished *M. intracellulare* from *M. chimaera*.

Sequencing for identification of *M. intracellulare* and *M. avium*. All isolates of presumptive *M. intracellulare* using nonsequencing methods underwent ITS region sequencing. The isolates had 100% identity to Min-A (*M. intracellulare*) or MAC-A (*M. chimaera*) (as the two type strains), and several sequences belonged to other MAC X species (11, 16, 21). A comparison of the base pair differences in the ITS region sequences between *M. intracellulare* and *M. chimaera* is shown in Table 4. Unlike the 16S rRNA gene that differed by only 1 bp substitution, the ITS region sequences differed by 19 bp substitutions (Table 4).

TABLE 2 Respiratory or sinus cultures of MAC initially identified as *M. intracellulare* by nonsequencing methods and then retested by ITS sequencing

Study no.	No. of *M. intracellulare* isolates identified by nonsequencing methods	No. (%) of isolates identified by ITS sequencing				Reference(s)
		M. avium	MAC X	*M. chimaera*	*M. intracellulare*	
1	1	1	0	0	0	6
2	3	0	0	0	3	L. Lande, unpublished data; 17
3	10	0	0	1	9	1
4[a]	40	0	0	3	37	R. J. Wallace, Jr., unpublished data
5	0	NA[b]	NA	NA	NA	2
Totals	54	1	0	4 (7.4)	49 (90.7)	

[a] Multiple isolates were available for all patients in this study.
[b] NA, not applicable.

The patient and water isolates of *M. chimaera* (identified by ITS sequencing) also underwent partial (initially 500 bp) 16S rRNA gene sequencing. This included the T→C base pair substitution at position 450 that distinguishes *M. intracellulare* from *M. chimaera* (16). All sequences were a 100% match to the GenBank sequence of *M. chimaera* submitted by Tortoli et al. (16) (accession no. NR_029003) and to the *M. chimaera* type strain sequenced as a control.

A total of 35 random *M. avium* isolates underwent ITS sequencing. All had Mav-A or Mav-B sequences typical of *M. avium*.

Respiratory isolates. A total of 54 respiratory cultures were identified as *M. intracellulare* by nonsequencing methods. By ITS sequencing, 49 isolates (90.7%) had the Min-A sequence of *M. intracellulare*, and 4 isolates (7.4%) had the MAC-A sequence of *M. chimaera* (Table 2).

Household water sampling. A total of 752 individual sites were sampled for the 73 patients from 80 households (Table 1). A total of 41 household water/biofilm isolates identified as *M. intracellulare* by nonsequencing methods underwent ITS and/or 16S rRNA partial gene sequencing, which revealed that the majority of these isolates (30/41, 73%) were *M. chimaera* or other MAC X species (8/41, 19.5%). Based on the ITS region sequencing, no isolate of *M. intracellulare* was recovered from any of the 752 sampled sites (Table 3).

DISCUSSION

This study indicates that environmental sources other than household water are the source of *M. intracellulare* nodular-type lung disease in the United States. More than 700 water plumbing sites from 71 patients from the Southwest, the Northeast, and more than 10 additional states were cultured for MAC. This makes the failure to recover *M. intracellulare* from household water samples in the United States not likely to be a function of either geography or inadequate sampling. There is no evidence that the decontamination procedure or the growth medium inhibits the growth of *M. intracellulare* (18). ITS sequence analysis detects only the most abundant microbial species. Thus, while ITS sequencing results failed to detect *M. intracellulare* in water samples, quantitative PCR sequencing needs to be done to definitively rule out its absence.

M. intracellulare is a recognized cause of MAC lung disease in the United States. Because species identification within the complex is not (yet) considered clinically important, it is usually not done except as part of prospective studies. Multiple studies of MAC lung disease have been done over the past 20 years at the UTHSCT with patients primarily from Texas and Louisiana. Species identification has been done in many of these studies. They have shown that 70% to 90% of MAC isolates from cases of nodular bronchiectatic disease and upper lobe fibrocavitary disease are *M. intracellulare* (23–25). (Some isolates from these older studies might have been *M. chimaera*, as all used nonsequencing species identification.) Few studies of the species of the MAC causing chronic lung disease that meet the ATS diagnostic criteria have been done in other areas of the United States, but *M. intracellulare* is considered an important cause of lung disease in these areas as well.

M. intracellulare is a major cause of MAC lung disease in sev-

TABLE 3 Species in household water cultures (one culture per site) initially identified as *M. intracellulare* and then retested by ITS sequencing compared to the number of isolates of *M. avium* recovered from the same samples

Study no.	No. of isolates identified by nonsequencing methods[a]		No. (%) of isolates identified by ITS sequencing				Reference(s)
	M. avium	*M. intracellulare*	*M. avium*	MAC X	*M. chimaera*	*M. intracellulare*	
1	20	4	3	0	1	0	6
2	64	8	0	3	5	0	L. Lande, unpublished data; 17
3	36	21[b]	0	5	16	0	1
4	18	8	0	0	8	0	R. J. Wallace, Jr., unpublished data
5	3	0	0	0	0	0	2
Total	141	41	3 (7.3)	8 (19.5)	30 (73.2)	0	

[a] Identification by either a DNA probe (AccuProbe; Hologic Gen-Probe Inc.) or 16S rRNA gene PCR (9, 10).
[b] Eighteen additional isolates were not tested.

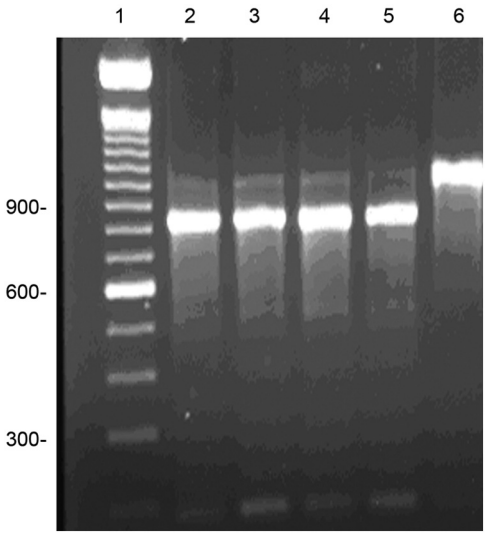

FIG 1 16S rRNA gene multiplex PCR comparing *M. intracellulare* and *M. chimaera* (9). The two species are indistinguishable by this method. Lane 1, 100-bp ladder; lane 2, *M. chimaera* type strain DSM 44623^T; lane 3, *M. chimaera* isolate from household water sample; lane 4, *M. intracellulare* type strain ATCC 13950^T; lane 5, *M. intracellulare* patient isolate; lane 6, *M. marseillense* isolate from household water sample. Molecular weight markers are shown to the left.

eral other countries. A recent study from Queensland, Australia, by Thomson compared NTM isolates obtained in 1999 and 2005 (26). There was a striking increase in *M. intracellulare* lung infections, with 79/97 (81%) of MAC lung disease cases being due to *M. intracellulare* and 19% due to *M. avium*. The method of species identification was not provided (26). A recent study of 590 Korean patients with MAC lung disease from 2000 to 2009 reported that 267 (45%) of the patients had *M. intracellulare* infections (27).

In contrast to the studies from the United States, Queensland, Australia, and Korea, previous studies from Japan reported that *M. avium* was the predominant cause of MAC lung disease (4). *M. avium* has also been shown to be the predominant or exclusive MAC species in water samples of residential bathrooms. In a 2009 study, Nishiuchi et al. found that 32/33 residential bathroom water MAC isolates were *M. avium*, and only one isolate was reported as *M. intracellulare* (utilizing a PCR method for species identification) (5). Nishiuchi et al. did report *M. intracellulare* in an earlier study from 3/9 bathrooms using the 16S to 23S internal spacer region for species identification (4). Neither of these studies reported the recovery of *M. chimaera* or other MAC X species from the water samples (4, 5). Thus, the characterization of the variability of MAC species populating water supplies in different parts of the world clearly warrants further investigation with more accurate sequencing techniques.

In 2009, Feazel et al. reported the use of 16S rRNA gene sequencing to determine the microbial composition of 45 shower sites collected from nine cities in the United States (3). Nontuberculous mycobacteria (NTM) were identified in approximately one-third of the samples, with the predominant species being *M. gordonae* and *M. avium*. *M. avium* was found in approximately 20% of all samples. Only two sampled sites, both in New York City, yielded clones of *M. intracellulare*, albeit in low numbers (3). In addition, the region of the 16S rRNA gene sequenced in this study excluded the base pair substitution at position 450 that distinguishes *M. chimaera* from *M. intracellulare*; therefore, these clones might have represented *M. chimaera* (3). The percentage of sites positive for NTM using 16S rRNA gene sequencing was similar to that with culture techniques (1, 6). Together, these studies

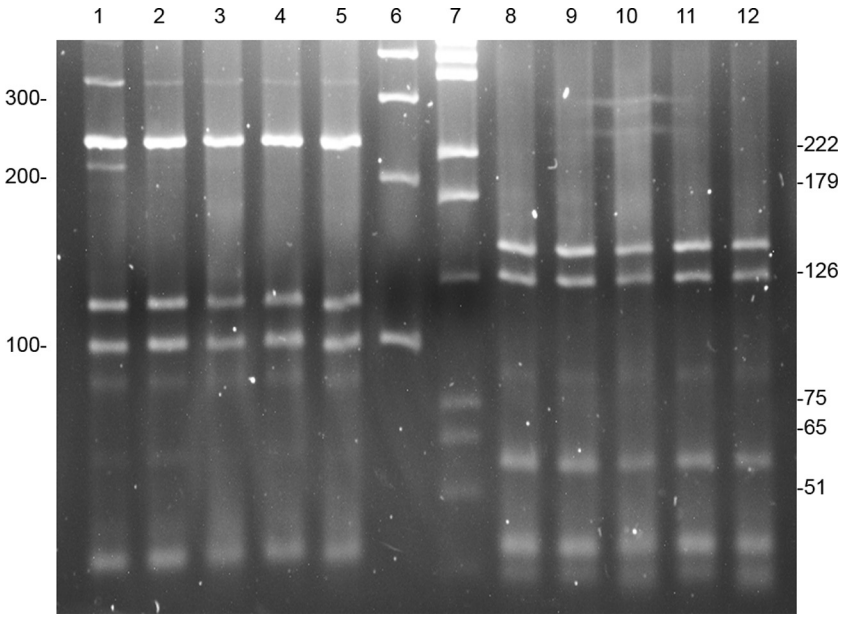

FIG 2 PCR restriction enzyme analysis (PRA) of the 441-bp Telenti fragment of the *hsp65* gene. The first five lanes include digests with BstEII (fragments of 235-115-100), and lanes 8 to 12 include the same isolates digested with HaeIII (fragments of 145-125-55). The digest patterns for *M. marseillense*, *M. chimaera*, and *M. intracellulare* are indistinguishable. Lanes 1 and 8, *M. marseillense*, household water; lanes 2 and 9, *M. chimaera*, household water; lanes 3 and 10, *M. intracellulare*, patient isolates; lanes 4 and 11, *M. chimaera* type strain DSM 44623^T; lanes 5 and 12, *M. intracellulare* type strain ATCC 13950^T; lane 6, 100-bp ladder (100, 200, 300, 400); lane 7, pGEM ladder (51, 65, 75, 126, 179, 222, 350). Molecular weight standard sizes are shown to the left and the right.

TABLE 4 Base pair differences in ITS region sequences between *M. intracellulare* (Min-A, -B, -C, and -D) and *M. chimaera* (MAC-A) and the MAC X species *M. colombiense* (MAC-U)

ITS region sequence[a]	Base pair differences with ITS sequevar of[b]:																				
	22	55	73	76	137	138	148	216	220	229	230	232	237	239	244	264	268	269	270	272
Min-A	T	C	G	C	G	A	A	G	A	A	G	G	T	C	G	C	G	G	T	T
Min-B	●	●	●	●	●	●	●	●	●	G	C	●	●	●	●	●	●	●	●	●
Min-C	●	●	●	●	●	●	●	●	●	G	T	●	●	●	●	●	●	●	●	●
Min-D	●	●	●	T	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●
MAC-A	C	T	A	●	A	G	T[c]	A	G	G	T	A[c]	C	T	A	T	A	A	C	C
MAC-U	●	T	A	●	●	●	●	A	G	A	T	●	C	T	A	●	●	●	●	●

[a] Note the similarity of the two MAC X species (*M. chimaera* and *M. colombiense*) and their dissimilarity with the four ITS sequences of *M. intracellulare*.
[b] ●, same base pairs as in Min-A.
[c] Base pair difference unique among the *M. avium* complex (12).

indicate that *M. intracellulare* is absent from U.S. household water systems, in contrast to the prevalence of *M. avium* (3).

As noted previously, the individual species in the *M. avium* complex are rarely reported clinically in the United States. When reporting clinical studies, most U.S. investigators use the nonsequencing methods that include a hybridization DNA probe assay, a 16S rRNA gene multiplex PCR, or restriction fragment length polymorphism (RFLP) analysis of the PCR product of amplification of the *hsp65* gene (1, 5, 9, 10, 20). This study points out the inadequacies of nonsequencing identification methods for closely related NTM species such as *M. intracellulare* and *M. chimaera* (16). Figures 1 and 2 and the results obtained here with the AccuProbe (Hologic Gen-Probe, Inc.) confirm the findings reported in the initial study of *M. chimaera* by Tortoli et al. (16).

Few environmental studies have been done for the MAC other than tests of household or hospital water (28), and these studies were done before sequencing was readily available. Soil would seem the most logical alternative source, and one study of aerosols of potting soil found *M. intracellulare* (identified by 16S rRNA PCR [9] and *hsp65* PCR amplification and endonuclease restriction fragment patterns [20, 29]). Those methods would not have distinguished *M. chimaera* from *M. intracellulare*. More environmental studies of potential sources of *M. intracellulare* responsible for nodular and cavitary lung disease are clearly needed and will require attention to the distinction between *M. intracellulare* and *M. chimaera*.

The ITS sequence of *M. chimaera* from a respiratory sample in the United States was first reported by Frothingham and Wilson in a 1994 study of the use of ITS to produce subgroups within the complex (21). Patient details were not provided. We are aware of only one report (published in 2009) of *M. chimaera* as a respiratory pathogen in the United States. The authors described a patient whose infection occurred in the setting of chronic obstructive lung disease. The DNA was sequenced from pathological lung samples, but the organism was not recovered by culture (30). There have been no additional reports of *M. chimaera* in the United States that we were able to be identified; furthermore, the current study is the first description of *M. chimaera* from environmental sources. The studies mentioned above suggest that household water might be the reservoir for *M. chimaera* lung disease. More studies are needed to determine the relative importance of *M. chimaera* as a pathogen and the relationship of household water/biofilm and sputum isolates and to determine whether previous case definitions for NTM lung disease utilized for other species of the MAC will identify disease due to this new species (16).

More broadly, the data support the notion that lumping mycobacteria into groups and/or complexes, here the *Mycobacterium avium* complex (now including four *M. avium* subspecies and at least eight other species), will obscure unique characteristics. Those characteristics include their ecology, epidemiology, virulence (*M. chimaera* might have reduced virulence) (31), and even susceptibility to antibiotics.

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