

Spread of Multidrug-Resistant Bacteria by Moth Flies from Hospital Waste Water System

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We documented and analyzed moth fly occurrence and spread of multidrug-resistant bacteria in a tertiary care hospital in Germany. The moth flies (*Clogmia albipunctata*) bred in the sewage system, then moved into the hospital, carrying biofilm and multidrug-resistant bacteria on their feet. Subsequently, the hospital developed a pest control protocol.

Hospital-acquired infections caused by multidrug-resistant (MDR) pathogens pose major challenges (1). Whereas the concept of transmission of pathogenic bacterial organisms through contact with medical staff is well established, other ways of spreading have not been sufficiently addressed. A tertiary care hospital in Germany observed sporadic outbreaks of MDR pathogens that could not be attributed to usual means of contamination. Concurrently, an increase in moth flies was observed.

Psychodidae, the family that encompasses moth flies, includes a few species that can cause severe health problems, including the species *Psychoda alternata* and *Clogmia albipunctata*. Both species occur in large numbers where poor hygienic conditions exist, such as in sewage treatment plants, hospital waste water systems, or other environments where microbial biofilms exist, and therefore are considered nuisance pests (2). Reports suggest that wounds attract adult moth flies (3) and larvae have even been reported in samples from tear ducts (3). *C. albipunctata* moth flies have become a severe source of insect

infestations in hospitals. One report (4) provides a summary of the permanent distribution of the species throughout Europe.

Observations of both moth flies and outbreaks of MDR bacteria in the hospital were not initially linked. However, a newly constructed operating room (OR) could not be opened for use for >2 years due to occurrence of moth flies. The results of this study suggest that spreading of MDR bacteria by moth flies could explain these outbreaks.

The Study

We performed microbiologic analysis of all biofilm samples and moth flies by matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry (Bruker, <https://www.bruker.com>) and Phoenix automated microbiology system (Becton Dickinson, <https://www.bd.com>) according to European Committee on Antimicrobial Susceptibility Testing (EUCAST, <https://eucast.org>) guidelines. To visualize biofilms we used fluorescence in situ hybridization protocol on sections of embedded samples or moth flies (Appendix, <https://wwwnc.cdc.gov/EID/article/26/8/19-0750-App1.pdf>). We considered pathogens to be MDR if nonsusceptible to ≥ 1 agent in 3 of the defined categories, extensively-drug-resistant (XDR) if nonsusceptible to ≥ 1 agent in all but ≤ 2 of defined categories, and pan-drug resistant (PDR) if nonsusceptible to all listed antimicrobials (5).

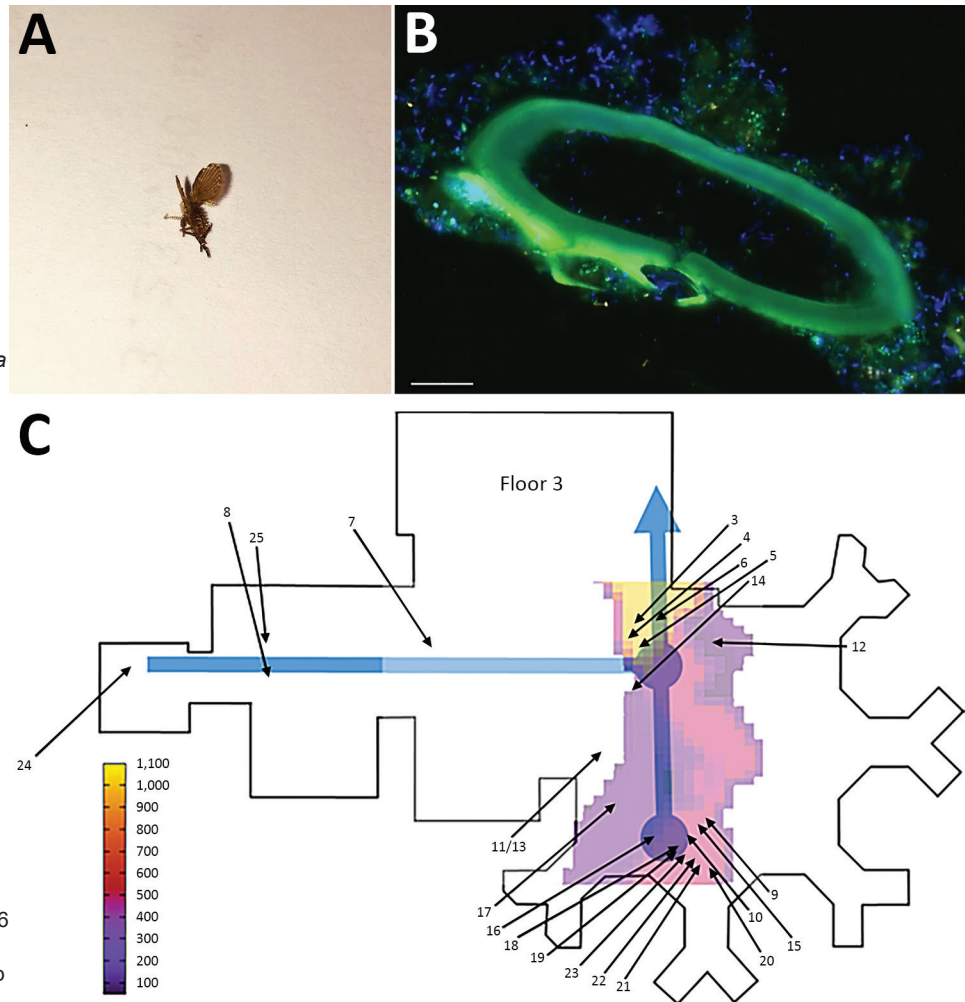
We identified the moth flies as *C. albipunctata* (Figure 1, panel A) and found that they had entered the hospital room from a forgotten shunt between the drains and the waste air system (Appendix). To confirm that sewage pipes contained bacterial biofilms and moth fly eggs, samples were taken from the pipes, fixed, and stained. Psychodidae eggs were found in the biofilm (Figure 1, panel B). Infestation with moth flies seemed to increase with proximity to the central drain (Figure

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Figure 1. Investigation of multidrug-resistant bacteria spread by moth flies via biofilm in a hospital, Germany. A) Magnified *Clogmia albipunctata* moth fly. The length of the corpus is 2.5 mm. B) Fluorescence in situ hybridization (FISH) from biofilm of a sewage pipe with a blind end in the operating room (OR) using the pan-bacterial FISH-probe EUB338 labeled with fluorescein isothiocyanate (green), *Pseudomonas aeruginosa* specific probe labeled with Cy3 (orange), and nucleic acid stain DAPI (Thermo Fisher Scientific, <https://www.thermofisher.com/>) (blue). The oval structure seen is consistent with an eggshell of Psychodidae, which is colonized by bacteria. C) Schematic map of floor -3 (topographically representative of the hospital building). Blue lines represent the main sewage pipe; heat map shows the frequency of *C. albipunctata* occurrence merged from all floors of the building; blue arrow indicates sewage system discharge from the hospital; black arrows indicate where moth flies were captured on level -3. Arrow numbers correspond to the numbers in Tables 1, 2; arrows 3–6 indicate the position of the closed OR. The gradients in the heat map (summarized over all floors) point to the yellow region, which is 1 floor above the central sewage collection point of the hospital. The central sewage lines were inspected; we found biofilm and multiple moth flies at all investigated points.



1, panel C). Furthermore, we observed that moth flies were able to pass through the water-filled siphon of a bedpan washer (Video 1, <https://wwwnc.cdc.gov/EID/article/26/8/19-0750-V1.htm>) and we identified extensive biofilm in the drains (Video 2, <https://wwwnc.cdc.gov/EID/article/26/8/19-0750-V2.htm>).

Fluorescence in situ hybridization and fluorescence microscopy on sections of embedded moth flies showed the presence of biofilms and bacteria on their feet (Figure 2). Not every moth fly caught was tested; however, representative moth flies from each identified location were tested. Furthermore, biofilm in sewage pipes revealed a kind of ecosystem consisting of moth fly larvae, vermicular, fungi, and bacteria (Appendix Figure 1). Subsequently we collected and microbiologically analyzed *C. albipunctata* and biofilm from different parts of the hospital (Tables 1, 2). Of the moth flies we analyzed microbiologically, we found

that 41.1% carried MDR or even XDR bacteria (Appendix). Overall, 43.9% of specimens were MDR or XDR.

We subsequently examined pest control options. Our first approach, removing biofilm from accessible pipes in the sewage system, did not successfully reduce or eliminate moth flies. Our second approach, mechanically and chemically cleaning all sinks and proximal sewage lines, also did not prevent periodic reoccurrence of moth flies. Our third approach was more successful. We flushed all sinks in the OR at the same time with 60°C hot water for 15 min/wk, daily during summer, which suppressed *C. albipunctata* moth flies in the OR but not in the rest of the hospital.

Conclusions

Infection with and colonization by MDR bacteria is an increasing challenge in health care (6). Psychodidae (moth flies) are small, 1–4 mm in size, and have been

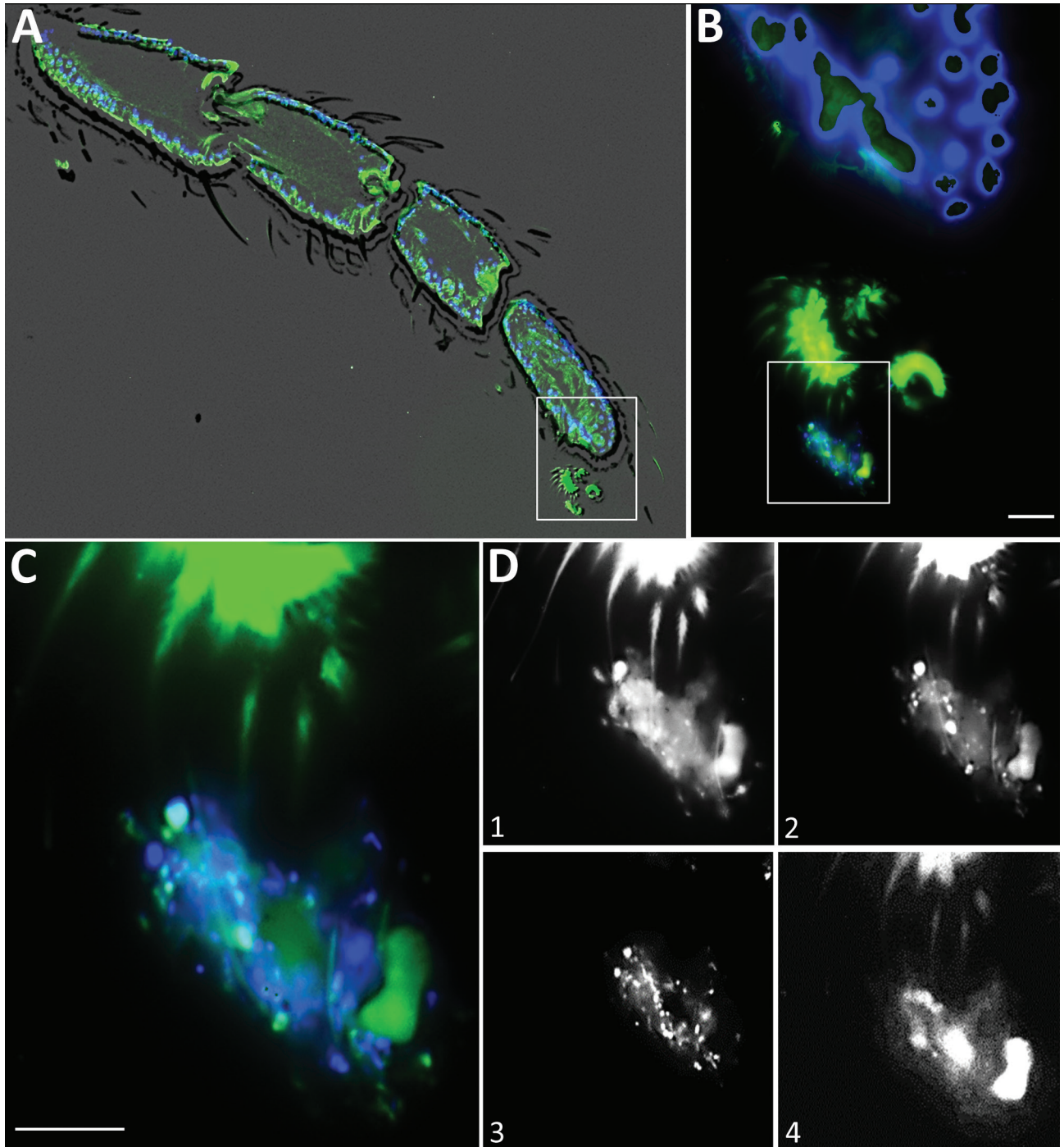


Figure 2. Fluorescence in situ hybridization (FISH) from a longitudinal section of a leg of a *Clogmia albipunctata* moth fly from a hospital in Germany. The fly was caught in the hospital, embedded, and stained (Appendix, <https://wwwnc.cdc.gov/EID/article/26/8/19-0750-App1.pdf>). A) Overview showing an overlay of the fluorescent images with a phase contrast to visualize the limbs of the legs. B) Higher magnification of the inset from panel A shows the anatomy of the tarsus and claws with an adjacent biofilm, which is stained by the bacterial probe (green) and DAPI (blue). C) Higher magnification of the inset from panel B shows the biofilm. Blue represents DAPI staining of DNA; bacteria were stained green with pan-bacterial FISH probe EUB338-FITC, *Enterobacteriales* stained orange with an *Escherichia coli*-specific FISH probe (data not shown), and NONEUB (nonsense EUB) probe labeled with Cy5 was used to exclude unspecific probe binding. D) Overlay of the DAPI and fluorescein isothiocyanate channel shows the biofilm with different bacterial morphotypes. Different planes of the z-stack in the green channel (pan-bacterial probe) of the identical microscopic field depicts the different claws embracing the biofilm (D1 and D2). D3 shows the DAPI filter-set only with the DNA of the bacteria, whereas D4 shows the autofluorescence in the Cy5 filter-set NONEUB probe.

Table 1. Occurrence of moth flies in a hospital, Germany*

No.	Psychodidae larvae/eggs	Count†	Type of room (comment)	Floor‡
1	Adult	1	Sewage line service opening (under OR 1)	-4
2	Larvae and eggs	>100	Sewage line service opening (200 m distant from OR 1)	-4
3	Adult	>50	OR 7§	-3
4	Adult	>500	Washroom, OR 7	-3
5	Adult	>50	Corridor, OR 7	-3
6	Adult	>50	Supply rooms for OR 7	-3
7	Adult	3	Corridor connecting ORs 1–6	-3
8	Adult	1	Corridor to ICU 58	-3
9	Adult	>20	Toilet (A3–40)	-3
10	Adult	>5	Doctor's room (A3–43) St 35	-3
11	Adult	>10	Toilet (G3–58)	-3
12	Adult	>10	OR dermatology (B3–61)	-3
13	Adult	>500	Washing room G3–62	-3
14	Adult	>500	Toilet A3–06	-3
15	Adult	>5	Supply room (A3–41)	-3
16	Adult and eggs	>500 (>100 eggs)	Shower (F3–07) St 35	-3
17	Adult	>500	Shower floor 35 (F3–31), patient room and corridor	-3
18	Adult	>10	Bathroom floor 34 (E3–07)	-3
19	Adult	>10	Shower floor 34 (E3–08)	-3
20	Adult	>10	Clean supply room floor 34 (E3–01)	-3
21	Adult	>10	Kitchen floor 34 (E3–02)	-3
22	Adult	>10	Staff room floor 34 (E3–04)	-3
23	Adult	>10	Doctor's room floor 34 (E3–05)	-3
24	Adult	>5	ICU floor 58 W3001	-3
25	Adult	3	OR 12 heart surgery¶	-3
26	Adult	>500	Sluice to hospital kitchen (S2–20)	-2
27	Adult	>500	Hospital kitchen toilets (S2–20a, b)	-2
28	Adult	>10	Supply room emergency department (R1–52)	-1
29	Adult	>10	Kitchen emergency department (R1–57)	-1
30	Adult	>10	Toilet emergency department (R1–55)	-1
31	Adult	>10	Patient rooms emergency department	-1

Patient numbers correspond to the numbers in Table 2 and Figure 1, panel C. ICU, intensive care unit; OR, operating room; St, suite.

†Count of Psychodidae larvae and adult flies during June 2016–October 2018.

‡Floor numbers are negative because the hospital is in part built on a hill so that Floor 0 is the most top level (Floor 0 had no moth fly observations).

§OR 7 had been closed for years until we eliminated the source of moth flies.

¶In OR 12 only 1 moth fly was found even after an intense search. From all immediately reported occasions 1–2 moth flies were caught and analyzed microbiologically (see Table 2 for results).

regarded as unharmful vermin except in highly sterile areas. Therefore, they have often been overlooked or ignored and not considered a high-consequence problem.

The results of our study suggest a change in this point of view is needed. If generalized to other hospitals, our findings indicate that *C. albipunctata* moth flies in hospitals, combined with MDR, XDR, or PDR (pandrug-resistant) bacteria in biofilms, pose an underestimated threat. The danger from this symbiotic system between moth flies and these bacteria results from moth fly eggs and larvae living in biofilm that is contaminated by a patient's bacterial flora. Furthermore, biofilms can rapidly grow and spread over distances kilometers in length (7) and are almost impossible to eradicate. In the third and fourth stages of development, larvae living in the biofilm can begin to move, thus overcoming the water barriers in showers, bathtubs, toilets, and other washing units. At this point, adult moth flies can enter the hospital (Video 1) and transport drug-resistant bacteria from the microbial flora of the biofilm into the hospital.

We frequently found *Stenotrophomonas maltophilia* on *C. albipunctata* moth flies and also in clinical samples from deep respiratory material, wounds, blood culture, urine, and bile. In 1 patient, for example, we found hospital-acquired *S. maltophilia* and a genetically identical strain in drains in a ward ≈250 m away (data not shown). Even though this evidence is scant, it does support our hypothesis.

In addition, low doses of antimicrobials excreted by patients can result in the quick development and spread of plasmids (resistance genes) and virulence factors in biofilms (8). This process might result in resistance developing not only in a patient's microbiota but also in hospital biofilm. Our observations suggest that the adult *C. albipunctata* moth flies can move freely throughout sewage systems and that they carry bacterial biofilm on their feet. Many authors have suggested the existence of missing links in polyclonal outbreaks and in other hard-to-explain observations (9,10). We hypothesize that moth flies in symbiotic combination with biofilms could, in part, explain one such observed

transmission. However, the findings of this study are limited by the moderate number of moth flies, which should be addressed in future investigations.

Currently, there are no proven strategies, including chemical methods, to prevent or eradicate moth flies in sewage systems. However, weekly or, during summer, daily flushing with hot water (60°C) for 15 min was sufficient to suppress the moth flies in our study. We propose a prevention protocol including flushing weekly or daily with hot water (60°C), mechanical removal of biofilms; deconstruction of unused siphons or replacement by heatable siphons; and checking for unexpected outlets, such as drill holes, from drains into

hospital rooms. These measures will not eliminate but might substantially suppress the problem. Once moth flies leave the drains, among the few available biofilms are patient wounds. Research has reported that adult moth flies are attracted to them, and *C. albipunctata* larvae have been found in wounds (11,12). Searching for moth flies and determining their microbial load might be advisable, especially if an unexpected bacterial outbreak occurs. Finally, our observations should be taken into account in the planning of hospital sewage systems in the future.

The authors declare no conflict of interest.

Table 2. Multidrug resistant bacteria on moth flies and biofilm in a hospital, Germany*

No.	Specimens tested	Location found	Species identified†	Can produce biofilm?	Resistance level‡	Macroscopic/histologic findings
1	Biofilm	Sewage line	<i>Achromobacter xylosoxidans</i>	Yes	Not classified	Biofilm, <i>Candida</i> , mucin, eggs
2	Biofilm	Sewage line	<i>Pseudomonas</i> spp. <i>Escherichia coli</i> <i>Lysinibacillus fusiformis</i>	Yes	Potential XDR§ MDR	Biofilm, cocoons, eggs
3	Moth fly	OR 7	<i>Bacillus</i> spp., <i>Citrobacter freundii</i>	Yes	MDR	NA
4	Biofilm	Endoscopy of sewage lines in washroom, OR 7	<i>Advenella</i> species	Yes	Potential XDR§	Extensive biofilm in all sewage lines, mucin, <i>Candida</i>
			<i>Bacillus</i> spp.	Yes		
			<i>Pseudomonas aeruginosa</i>	Yes		
			<i>Stenotrophomonas maltophilia</i>			
	Moth fly	Washroom OR 7	Sterile	NA	ND	NA
5	Moth fly	Corridor OR 7	<i>P. mosselii</i> <i>S. maltophilia</i>	Yes	Potential XDR§	NA
7	Moth fly	Corridor OR 1-6	<i>Bacillus</i> spp. <i>B. megatariume</i>	Yes Yes	ND	NA
8	Moth fly	Corridor ICU CS	<i>Bacillus</i> spp.	Yes		NA
9	Moth fly	Toilet (A3-40)	<i>S. maltophilia</i>		Potential XDR§	
	2 moth flies	Toilet (A3-40)	Sterile	NA	ND	NA
10	Moth fly	Doctors room (A3-43)	<i>Bacillus</i> spp.	Yes	ND	NA
	Moth fly	Doctors room (A3-43)	<i>P. nitroreducens</i> <i>B. cereus</i> <i>B. thuringiensis</i>	Yes Yes Yes	ND	NA
14	Moth fly	Toilet (A3-06)	<i>Bacillus</i> spp. <i>B. cereus</i> <i>Chryseobacterium indologenses</i>	Yes	ND	NA
16	Biofilm	Shower drain	<i>B. cereus</i> <i>Bacillus</i> spp. <i>P. putida</i>	Yes	ND	Biofilm, eggs, <i>Candida albicans</i>
	Moth fly	Shower (F3-07)	<i>Bacillus cereus</i> <i>L. sphaericus</i> <i>Staphylococcus epidermidis</i>	Yes	ND	NA
	2 moth flies	Shower (F3-07)	<i>S. maltophilia</i> <i>B. thuringiensis</i>	Yes	Potential XDR	NA
17	Moth fly	Shower (F3-31)	<i>Enterococcus faecium</i> (<i>vanA</i>) <i>Bacillus</i> spp. <i>S. maltophilia</i>	Yes	Possible XDR¶ Potential XDR§	NA
	Moth fly	Shower (F3-31)	<i>E. faecium</i>	Yes	MDR	NA
24	Moth fly	ICU floor C	Sterile	NA	ND	NA

*Numbers at left are patient numbers, which correspond to the numbers in Table 1 and Figure 1, panel C. CS, cardiosurgery; C, cardiology; ICU, intensive care unit; MDR, multidrug resistant; OR, operating room; NA, not applicable; ND, not detected; XDR, extensively drug resistant.

†Microbiology, histology, and fluorescence in situ hybridization findings in the investigated specimens. Note that not every moth fly from the Table 1 count column was analyzed.

‡Resistance levels defined in Magiorakos et al. (13).

§Potential XDR means that the international standards have not been applied to the bacterium *S. maltophilia*. Additional details are available in the Appendix (<https://wwwnc.cdc.gov/EID/article/26/8/19-0750-App1.pdf>).

¶Possible XDR means that it could be XDR but not all antimicrobial categories were tested. Additional details are available in the Appendix.

About the Author

Dr. Thomas Rupprecht is a lecturer at the University Erlangen Nürnberg and medical director at the hospital Bayreuth. His research interests are complex human and environmental biosystems, their influence on disease occurrence and treatment, and mathematical modeling in both.

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Spread of Multidrug-Resistant Bacteria by Moth Flies from Hospital Waste Water System

Appendix

Material and Methods

Identification of moth fly sources

We completely isolated the operating room (OR) from the rest of the building by fly-proof enclosure. We closed and reopened all input and output connections (water pipes, air conditioning, drainage pipes, sewage lines) step by step to identify the source of moth flies. We also closed all sinks in the OR with tape. We did this to catch moth flies on the sticky site of the tape and thereby identify all sinks where moth flies might have left the siphons. The fluff filter of the waste air system was used as a sentinel for moth flies. Members of the task force did weekly documented inspections to identify the moth fly source and to examine the efficiency of pest control measures. In the rest of the hospital, the medical staff was advised to search for and to report the presence of moth flies. Finally a heat map was calculated (Gnuplot bilinear interpolation, <http://www.gnuplotting.org/>) to show the gradients of the count of fly occurrence and to locate the primary source.

Taxonomic identification of moth flies

The taxonomic identification of *Clogia albipunctata* was done by one of the author (R.W.) who is an expert on Psychodidae. For the identification moth flies were fixed in 80% ethanol and analyzed by microscope by 200x magnification. The size, wing shape, coloration, and genitalia were analyzed and the moth flies were identified by the expert knowledge of R.W. as *C. albipunctata* (1). For further illustration we depict a life cycle of *C. albipunctata* in Appendix Figure 2.

Collection of moth flies and biofilm samples and analysis

Moth flies were collected and characterized histopathologically and microbiologically using classical culture techniques and fluorescence in vitro hybridization (FISH) analysis. Furthermore, the central occurrence sites of the sewage lines were inspected and sampled

distally to the water barriers (siphons) by endoscopy (Ambu aScope, <https://www.ambu.com/>).

Microbiologic Culture Techniques

All specimens were investigated by classical microbiologic cultures. Hence, moth flies were captured in sterile tubes with 25 mL PP container yellow screw caps (Sarstedt, <https://www.sarstedt.com/>), flooded with 8 mL brain-heart-infusion bouillon (BD, <https://www.bd.com/>), and then incubated at 35°C for 20 h. Subsequently, 50 µL of brain-heart-infusion was plated on selective agar for VRE-Agar (MAST Diagnostica, <https://mast-group.com/>), MRSA-Agar (MAST Diagnostica), ESBL-Agar (bioMerieux, <https://www.biomerieux.com/>), and TSA (BD) subsequently plates were incubated for additional 20 h. Bacterial species were identified by MALDI-TOF (Bruker, <https://www.bruker.com/>), antimicrobial resistance was tested applying the Phoenix-System (BD) and result interpretation was done according to EUCAST clinical breakpoints (www.EUCAST.org). Antimicrobial resistance genotype for VRE was determined applying *vanA/B*-PCR (Cepheid, <https://www.cepheid.com/>).

Pest control measures

To find appropriate and cost-effective pest control measures we took advantage of the closed OR which was a well-controlled setting. In addition, only the task force members were authorized to access the operation room and this was controlled by sealing all rooms after each inspection (weekly). The untouched seals were photo documented at the beginning of the next inspection.

Our first approach to pest control, mechanically removing biofilm from accessible pipes in the sewage system, did not successfully reduce or eliminate moth flies. Our second approach, mechanically and chemically cleaning all sinks and proximal sewage lines with pyrethroid insecticide, also did not prevent periodic reoccurrence of moth flies. Our third approach was more successful. We flushed all sinks in the OR at the same time with 60°C hot water for 15 min/wk (daily during summer) which suppressed *C. albipunctata* in the OR but not in the rest of the hospital.

Definitions of Multidrug-Resistant-Organism

We used the definitions in Magiorakos et al. (2), which were developed from a consensus of international experts from the US Centers for Disease Control and Prevention and the European Center for Disease Prevention and Control. Definitions were set only for

the most common bacteria and include definitions for multidrug-resistant (MDR), extensively drug-resistant (XDR) and pandrug-resistant (PDR) bacteria. In brief, we considered pathogens to be MDR if nonsusceptible to ≥ 1 agent in 3 of the defined categories, extensively-drug-resistant (XDR) if nonsusceptible to ≥ 1 agent in all but ≤ 2 of defined categories, and pan-drug resistant (PDR) if nonsusceptible to all listed antimicrobials. “Possible” is used to modify MDR, XDR or PDR means that the drug resistance definition could be met, but not all listed antimicrobials have been tested.

However, neither *Stenotrophomonas maltophilia* nor *Pseudomonas* spp., which we found in sewage lines as well as on moth flies, are included in this definition. Because we tested according to EUCAST rules and for *S. maltophilia* EUCAST provides only breakpoints for co-trimoxazole, it is difficult to classify this bacterium. To address this problem, we also list the breakpoints for all tested antimicrobials which demonstrates that, except for co-trimoxazole, none is susceptible when interpreted by Clinical & Laboratory Standards Institute criteria. In addition, *S. maltophilia* is MDR even though only in part these resistances are acquired. To address this problem we classified the *S. maltophilia* isolates as “potential” XDR, acknowledging that this is more a proposal than a fact. The same descriptor (potential XDR) was applied to *Pseudomonas* spp. as the international classification is defining criteria only for *P. aeruginosa*.

Fluorescence in vitro hybridization

Biofilm samples and moth flies were fixed in FISH-fixation solution (MoKi Analytics GmbH, Berlin, Germany) at 4°C for 3–6 days, embedded in cold polymerizing methacrylate resin and sectioned in 2 μ m sections as described (3). Sections were first screened with the pan-bacterial, 16S rRNA directed probe EUB338 (4), which detects most bacteria and EUK516 for detection of Eukarya (5). To exclude unspecific probe binding, positive FISH signals were reviewed using the NONEUB (nonsense EUB) probe NON338 (3). For genus- or species-specific detection of bacteria we used probes specific for *P. aeruginosa* (PSMG) (6), *Enterobacteriales* (EC1531) (7), and *Staphylococcus* (STAPHY) (6,8). The nucleic acid stain DAPI (4',6-diamidino-2-phenylindole) was applied as a counterstain to visualize microorganisms and eukaryotic cell nuclei.

Supplemental results

Moth fly source in the OP

We identified 2 possible ways of moth fly invasion which were the air conditioning and the drains. However, biofilm, which is essential for moth flies, was only found in the sewage lines. Most likely there was a shunt between the sewage system and the waste air system due to a removed autoclave. After the shunt was closed, we detected no further moth flies in the OR (operation room). Furthermore, we noticed that the prolonged shut down of the OR facilitated moth flies to leave the drains through sinks into the OR (Video 1, <https://wwwnc.cdc.gov/EID/article/26/8/19-0750-V1.htm>). Closing sinks with tape proved that moth flies escaped the sewage pipes at all sinks in the OR because several moth flies were caught on the sticky side of the tape. It was concerning that moth flies can leave the drain, where MDR and XDR bacteria frequently occur. For this reason, we analyzed moth flies and sewage pipes for MDR, XDR, and PDR pathogens.

Moth fly identification

The moth flies were classified as male and female *C. albipunctata* (Figure 1, panel A).

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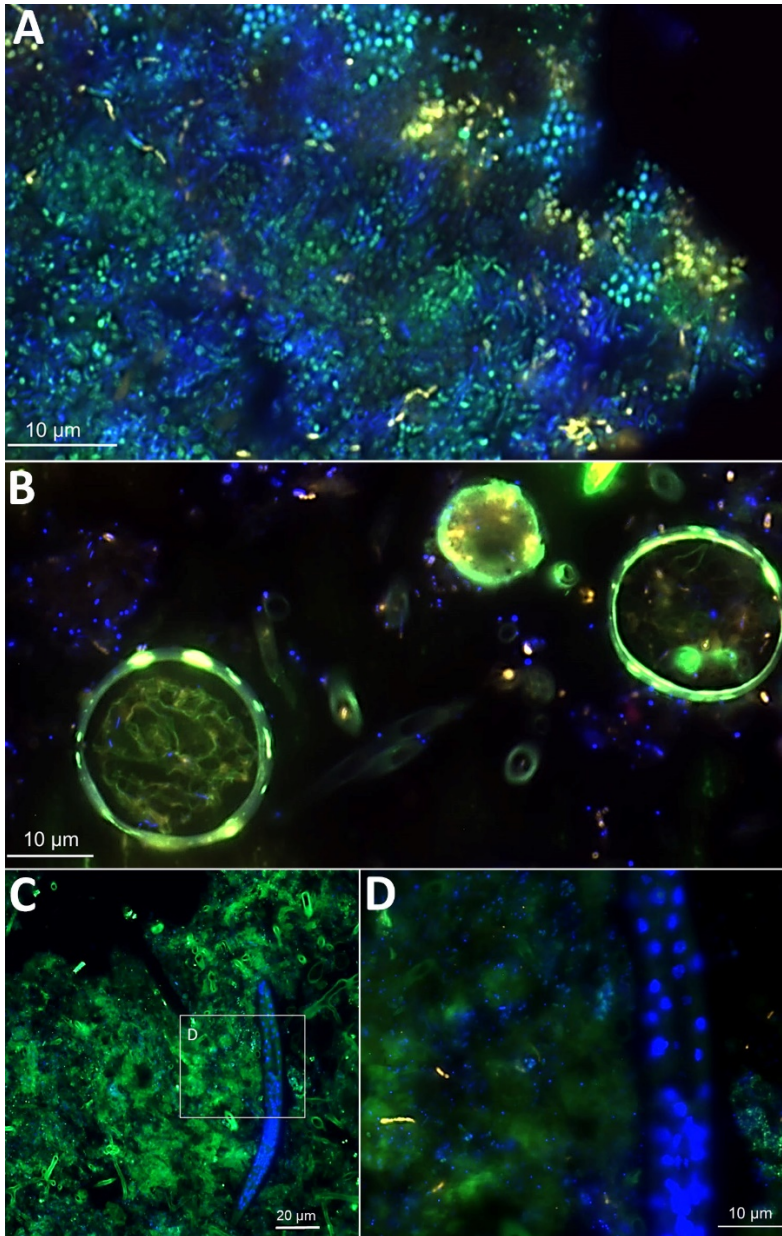
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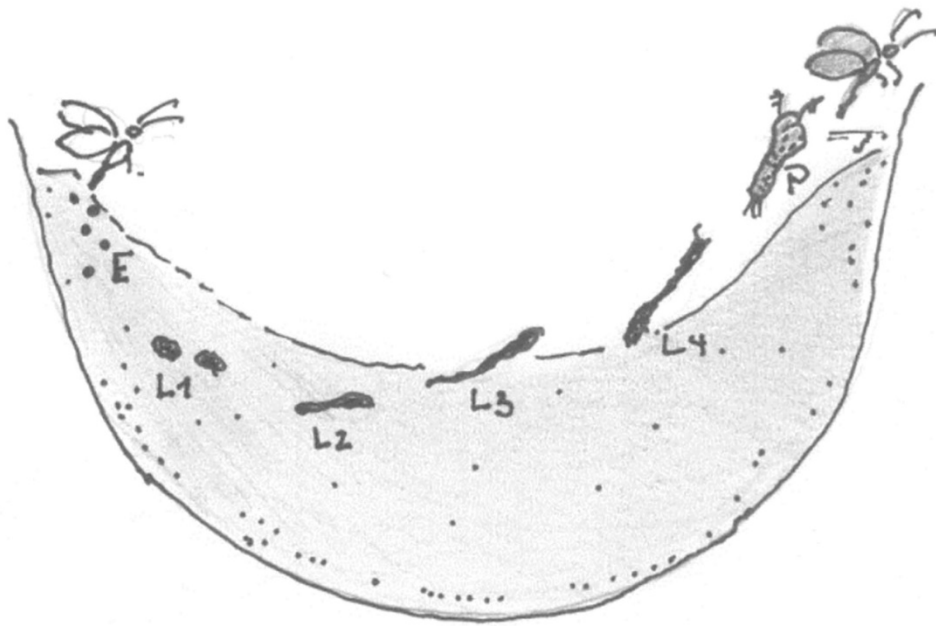
Appendix Table. Resistance phenotypes of bacteria*

Bacteria	<i>Stenotrophomonas maltophilia</i>		<i>Pseudomonas</i> spp.		<i>Escherichia coli</i>		<i>Citrobacter freundii</i>		VRE	
	MIC	C	MIC	C	MIC	C	MIC	C	MIC	C
Antimicrobials										
Penicillin G	†	‡	†	‡	†	‡	†	‡	>0.25	R‡
Flucloxacillin	†	‡	†	‡	†	‡	†	‡	>2	R‡
Erythromycin	†	‡	†	‡	†	‡	†	‡	>4	R‡
Clindamycin	†	‡	†	‡	†	‡	†	‡	>1	R‡
Vancomycin	†	‡	†	‡	†	‡	†	‡	>8	R§
Teicoplanin	†	‡	†	‡	†	‡	†	‡	>8	R§
Linezolid	†	‡	†	‡	†	‡	†	‡	1	S¶
Daptomycin	†	‡	†	‡	†	‡	†	‡	2	R§
Quinopristin/ Dalfopristin	†	‡	†	‡	†	‡	†	‡	1	S¶
Ampicillin	>8	R‡	>8	R‡	>8	R§	>8	R§	>16	R§
Ampicillin/ Clavulanic acid	>32/2	R‡	>32/2	R‡	†	R‡	†	R§	†	R§
Ampicillin/ Sulbactam	†	R‡	†	†	†	R‡	†	R§	†	R§
Tircarcillin/ Clavulanic acid	†	‡	>64/2	R§	32/2	R§	>64/2	R§	†	R§
Piperacillin	>64	R‡	8	I‡	8	S‡	>64	R‡	>64	R§
Piperacillin/ Tazobactam	>64/4	R§	>8/4	I§	8/4	S‡	8/4	S¶	16/4	R§
Cefuroxime	>8	R‡	>8	R‡	>8	R§	†	R§	†	R‡
Ceftriaxone	>4	R‡	>4	R‡	>4	R§	>4	R§	†	R‡
Ceftazidime	16	R§	4	I§	16	R§	>16	R§	†	R‡
Cefepime	†	§	16	R§	16	R§	<1	S	†	R‡
Meropenem	>8	R§	0.5	S§	<0.125	S¶	0.25	S¶	†	R‡
Imipenem	>8	R§	>8	R§	<0.25	S¶	1	S¶	>8	R‡
Aztreonam	†	‡	>16	R§	>16	R§	>16	R§	†	‡
Ciprofloxacin	>1	R§	>1	R§	<0.25	S¶	>1	R§	>1	R§
Levofloxacin	>2	R§	>2	R§	≤0.5	S¶	>2	R§	1	R§
Cotrimoxazol	2/38	S¶	>4/76	R§	<1/19	S¶	<1/19	S¶	†	R‡
Gentamicin	4	R§	>4	R§	<1	S¶	<1	S¶	>4	R§
Tobramycin	4	R§	>4	R§	<1	S¶	<1	S¶	4	R§
Amikacin	8	R§	8	S§	<4	S¶	<4	S¶	†	R§
Fosfomycin	>128	R§	>128	R§	<16	S¶	<16	S¶	>64	R‡
No. AMR groups	6		7		6		6		6	
No. remaining AMS groups	1		0		5		5		2	
Antimicrobial groups not tested	2		1		5		5		3	
Interpretation	Potential XDR*#		Potential XDR*#		MDR#		MDR#		Possible XDR#	

*Term "potential XDR" introduced to classify multidrug-resistant *S. maltophilia* and *Pseudomonas* spp., which were not addressed in the international classification but do show extensive resistance phenotype and would fulfill the criteria of an XDR according to the definition for *Pseudomonas aeruginosa*. AMR, antimicrobial resistant; AMS, antimicrobial sensitive; C, category; I, increased exposure; MDR, multidrug resistant; MIC, minimum inhibitory concentration; R, resistant; S, sensitive; VRE, vancomycin-resistant *Enterococci*; XDR, extensively drug resistant
†No breakpoint for this antibiotic for the bacterium because of insufficient evidence or does not work for gram-negative bacteria
‡Not part of the classification for this bacterium
§Antimicrobial group has ≥1 AMR substance
¶Antimicrobial group AMS
#MDR determined according to EUCAST clinical breakpoints (version 10.0); XDR according to EUCAST classification and our extension



Appendix Figure 1. Fluorescence in situ hybridization (FISH) from a blind sewage pipe in the operation theater (No. 2 in Table 2) using the pan-bacterial FISH-probe EUB338 labeled with FITC (B, green), *Pseudomonas aeruginosa*-specific probe labeled with Cy3 (orange), and nucleic acid stain DAPI. (A) shows the rich biofilm with different morphotypes and microcolonies, including numerous *P. aeruginosa* cells scattered throughout the biofilm. (B) Biofilm sample taken from the sewage pipes (No. 16 in Table 2, shower sink,) with several eggs (not from *Clogmia albipunctata*). DNA is stained blue by DAPI, EUB338-Cy3 (orange) as pan-bacterial staining, Eukara were stained green (EUK516-FITC), negative binding control NON338-Cy5 (magenta, data not shown). (C) FISH image showing a mature biofilm sample taken from the sewage pipes (No.4 in Table 2, endoscopy of sewage lines, Table 2) with hyphae and a worm, most likely a nematode. With the green filter set, auto-fluorescent hyphae are visible in the biofilm material in the overview. The nematode is distinguishable by the nucleic acid stain DAPI. (D) At higher magnification *P. aeruginosa* is visible, detected by a specific Cy3-labeled FISH-probe (orange).



Appendix Figure 2. Life cycle of *Clogmia albipunctata*. The adult female moth fly lays up to 300 eggs (E). Larvae develop in 4 stages (L1–L4); during L3 and L4 they can move in the biofilm and water, fed by organic substances in the film. Moth fly pupae (P), the last larval stage, migrate to the water surface from where the new moth fly leaves the cocoon. The whole cycle lasts ≈ 3 –4 weeks, depending on the environmental temperature (1,9).