Antimicrobial resistant and enteropathogenic bacteria in ‘filth flies’: a cross-sectional study from Nigeria

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‘Filth flies’ facilitate the dispersal of pathogens between animals and humans. The objective was to study the intestinal colonization with antimicrobial resistant and enteropathogenic bacteria in ‘filth flies’ from Nigeria. Flies from Southern Nigeria were screened for extended-spectrum β-lactamase producing Enterobacterales (ESBL-E), Staphylococcus aureus, Salmonella sp., Shigella sp., Campylobacter sp. and Yersinia enterocolitica by culture. ESBL-E were tested for blaSHV, blaCTX-M and blaTEM. S. aureus was screened for enterotoxins. Spa typing and multilocus sequence typing (MLST) was done for S. aureus and MLST for Escherichia coli. Of 2,000 flies, 400 were randomly collected for species identification. The most common species were Musca domestica (44.8%, 179/400), Chrysomya putoria (21.6%, 85/400) and Musca sorbens (18.8%, 75/400). Flies were colonized with S. aureus (13.8%, 275/2,000) and ESBL-E (0.8%, 16/2,000). No other enteropathogenic bacteria were detected. The enterotoxin sei was most common (26%, 70/275) in S. aureus, followed by sea (12%, n = 32/275). Four S. aureus isolates were methicillin resistant (mecA positive, t674 and t5305, ST15). The blaCTX-M (n = 16) was the most prevalent ESBL subtype, followed by blaTEM (n = 8). ‘Filth flies’ can carry antimicrobial resistant bacteria in Nigeria. Enterotoxin-positive S. aureus might be the main reason for food poisoning by ‘filth flies’ in the study area.

Diptera (true flies) are the most abundant and diversified endopterygota (or holometabola) of the insect order, with more than 180 families of about 158,000 described species1,2. ‘Filth flies’ (diptera) are universal, ubiquitous, coprophagic and synanthropic (living in close association with humans) insects that breed in garbage, animal and human faeces3. Known to serve as vectors of many pathogens, house flies can disperse pathogens through a flight distance of about 7 km between animals and humans6.

Flies transmit pathogens through three routes: mechanical translocation from the exoskeleton, regurgitation and defecation8. During feeding, flies can either pick up pathogens on its exoskeletal surfaces or ingest fluids contaminated with pathogens. Ingested pathogens can multiply in the crop (a blind sac of the digestive tract in higher flies) and after regurgitation which coined the term of “bioenhanced transmission”5.

Antimicrobial resistance (AMR) affects both humans and animals and antimicrobial resistant bacteria can be transmitted between animals and humans in both direction. This challenge is considered in the “one Health” approach. The most widespread resistance mechanisms in Enterobacterales is based on plasmid-mediated production of extended spectrum β-lactamases (ESBL) which hydrolyse β-lactam rings, thereby reducing the efficacy of cephalosporins and monobactams6. Flies are important reservoirs and vectors of antimicrobial resistant bacteria (such as methicillin resistant Staphylococcus aureus, ESBL-producing Enterobacterales [ESBL-E])1,5. One short report suggests that antimicrobial resistant bacteria can also be detected in flies (n = 25) in sub-Saharan Africa8. However, the true burden of AMR in ‘filth flies’ in Africa is unknown and it is currently unclear which fly species are the main vectors of ESBL-E and enteropathogenic bacteria. Therefore, the objective of this study was to analyse the colonization rates of ‘filth flies’ from Southern Nigeria with ESBL-E, S. aureus and other enteropathogenic bacteria and to identify those fly species which are mainly colonized with these target organisms.

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Results

Sampling sites. In total, 109 sites were sampled, with a total area cover of 15,075,000 m² (Fig. 1). A total of 2,000 flies were captured with a mean number of 18.3 (± SD 5.8) flies per sampling spot. Approximately 40 flies were caught per hour. The mean (± SD) atmospheric parameters were a temperature of 26 °C (± 1.6), relative humidity of 86.5% (± 6.2), windfall of 5.6 Beaufort (± 1.4), air pressure of 1012.4 hPa (± 1.3) and 13 sunshine hours (± 0.4). The sampled sites were urban (8.3%, 9/109), semi-urban (61.4%, 67/109) and rural (30.3%, 33/109).
| Environmental factors on day of sampling | Sample sites | Detection of S. aureus (n = 45), | No detection of S. aureus (n = 64), | Crude OR (95% CI)a | Crude p-valuea | Adjusted OR (95% CI)b | Adjusted p-valueb |
|-----------------------------------------|--------------|---------------------------------|---------------------------------|------------------|----------------|------------------|----------------|
| Animal faeces [n (%)]                  | 23 (51.1)    | 26 (46.6)                       | 1.5 (0.7–3.2)                  | 0.28             | NA             | NA               | NA             |
| Rural [n (%)]                          | 9 (20)       | 0 (0)                           | NA                             | NA               | NA             | NA               | NA             |
| Semi-urban [n (%)]                    | 15 (33.3)    | 52 (81.3)                       | 0.2 (0.1–0.4)                  | < 0.001          | 0.07 (0.01–0.4) | 0.001            | NA             |
| Mean temperature [°C (± SD)]          | 12 (4.7)     | 12 (12.7)                       | 0.5 (1.0–1.2)                  | 0.002            | 0.6 (0.4–1)    | 0.3              | NA             |
| Air pressure [hPa (± SD)]             | 1012.9 (1.3) | 1012.1 (1.2)                    | 1.6 (1.2–2.2)                  | 0.003            | 1.0 (0.9–1.2)  | 0.1              | 0.04           |
| Sunshine hours [h (± SD)]             | 12.9 (0.3)   | 12.7 (0.5)                      | 0.6 (1.5–19.9)                 | 0.01             | 1.4 (0.2–8.6)  | 0.8              | NA             |

Table 1. Association of *Staphylococcus aureus* in flies with environmental factors. SD standard deviation.

- aFrom an univariable analysis.
- bFrom a multivariable logistic regression analysis with a stepwise backward elimination. All environmental factors potentially associated with the detection of *S. aureus* in flies (p < 0.25) were entered in the multivariable model.

33/109). Within a 10 m radius of each sampling site (n = 109), we recorded decomposing organic matter (79.8%, 87/109), refuse dump (84%, 92/109) and animal faeces (49%, 49/109, Table 1).

### Intestinal culturome of flies.

The most common species of the intestinal culturome of 82 randomly selected flies were *Bacillus cereus* (73%, 60/82), followed by *Enterococcus faecalis* (27%, 22/82), *Enterococcus faecium* (24%, 20/82), *Clostridoides tertium* (21%, 17/82), *Bacillus licheniformis* (11%, 9/82), *Bacillus subtilis* (11%, 9/82), *Enterococcus hirae* (11%, 9/82) and others (70%, 57/82, Figure S1).

### *S. aureus.*

A total of 275 flies (13.8%) from 45 sites were colonized with *S. aureus*. Since *S. schweitzeri* is frequent in African wildlife, we tested if some isolates were misidentified by MALDI-TOF as *S. aureus*. All isolates were *nuc* positive and harboured the 160 bp-fragment of NRPS, thus ruling out *S. schweitzeri* in our collection. The most predominant *spa* type was 9674 (98%, 270/275) followed by singular occurrences of t1980, t5305 and t6313 (0.4%, 1/275 each, two isolates were not *spa* typeable). The four distinctive *spa* types belonged to MLST ST15 and showed similar *spa* repeat patterns (674: 07-34-12-23-02-12, 1980: 07-34-12-23, 5305: 07-34-12-23-02-12, 6313: 07-34-12-23-12-23). Since the majority of isolates belonged to 674, WGS was used to increase the discriminatory power in order to distinguish these isolates (Fig. 2). All isolates belonging to 674 differentiated by ≤3 alleles from the most closely related isolate.

The predominant antimicrobial resistance was against clindamycin (8.4%, 23/275) followed by tetracycline (2.2%, 6/275), penicillin (1.5%, 4/275), oxacillin (1.5%, 4/275), rifampicin (1.5%, 4/275), fusidic acid (0.8%, 2/275) and trimethoprim/sulfamethoxazole (0.4%, 1/275). All *S. aureus* isolates were susceptible to fluoroquinolones, macrolides, glycopeptides, gentamicin, linezolid, tigecycline and daptomycin. Four isolates were MRSA-positive, t5305 (n = 1), t674 (n = 3). They were found in two different sampling spots (05°56'34" N, 7°28'04" E and 06°26'04" N, 7°28'80" E). All isolates were negative for lukF-PV/lukS-PV and all penicillin-resistant isolates (1.5%, 4/275) were positive for *blaZ*. *S. aureus* isolates were positive for *chp* (100%, 275/275) and *scn* (97%, 268/275) while none carried sak (0%, 0/275). The staphylococcal enterotoxin gene sei was predominant (26%, 70/275) followed by *sea* (12%, 32/275). Other staphylococcal enterotoxins (*sec, sed, see, sef, seg* and *seh*) were not detected.

While *S. aureus* positive flies were less likely detected in semi-urban settings, a lower mean temperature, higher air pressure and a lower wind force were environmental factors that favoured the detection of flies with an intestinal colonization of *S. aureus* (Table 1).

The fly species that carried *S. aureus* isolates were less likely detected in semi-urban settings (6.4%), followed by *Bacillus* (8.4%, 23/275), *Clostridium* (2.2%, 6/275), *Pseudomonas* (1.5%, 4/275) and *Escherichia coli* (1.5%, 4/275). The staphylococcal enterotoxin gene *sei* was positive, t5305 (n = 1), t674 (n = 3). They were found in two different sampling spots (05°56'34" N, 7°28'04" E and 06°26'04" N, 7°28'80" E). All isolates were negative for lukF-PV/lukS-PV and all penicillin-resistant isolates (1.5%, 4/275) were positive for *blaZ*. *S. aureus* isolates were positive for *chp* (100%, 275/275) and *scn* (97%, 268/275) while none carried sak (0%, 0/275). The staphylococcal enterotoxin gene sei was predominant (26%, 70/275) followed by *sea* (12%, 32/275). Other staphylococcal enterotoxins (*sec, sed, see, sef, seg* and *seh*) were not detected.

### ESBL-E.

A total of 16 flies (8.8%) collected from seven sites (6.4%) carried ESBL-E (Fig. 1) and were found mostly within a radius of 2.0 km of the Abia State University Hospital (5° 49' 278" N, 7° 23' 772" E). ESBL-producing *E. coli* (n = 15) was predominant followed by *Enterobacter cloacae* complex (n = 1). ESBL-producing *E. coli* were resistant to ceftriaxone (100%, n = 15/15), ceftazidime (93%, n = 14/15), aztreonam (100%, n = 15/15), and...
ciprofloxacin (53%, n = 8/15) and trimethoprim-sulfamethoxazole (100%, n = 15/15). All ESBL-E were susceptible to piperacillin/tazobactam, carbapenems, amikacin, tigecycline, fosfomycin and colistin.

The most frequent ESBL group was *bla*\textsubscript{CTX-M} (100%, 16/16), followed by *bla*\textsubscript{TEM} (33%, 8/16) with no occurrence of *bla*\textsubscript{SHV}. CTX-M-15 was the most predominant subgroup (10/16), followed by CTX-M-1 (5/16) and CTX-M-27 (1/16). Among TEM beta-lactamases, TEM 1 was predominant (7/8) followed by TEM 116 (1/8). All ESBL isolates were negative for the *bla*\textsubscript{CMY-2} genes.

The majority of ESBL-producing *E. coli* were not grouped in any phylogroup and were consequently subjected to MLST (ST10 [n = 5], ST 617 [n = 2], and singular occurrences of ST40, ST44, ST46, ST218, ST443 and ST940, Fig. 3). Only two isolates belonged to phylogroup D (n = 1) and B2 (n = 1).

The predominant fly species that carried ESBL-E were *M. domestica* (44.0%, 7/16), *M. sorbens* (38.0%, 6/16), *C. putoria* (12.0%, 2/16) and *S. africa* (6.0%, 1/16).

**Enteropathogenic bacteria.** None of the flies carried *Salmonella* sp., *Shigella* sp., *Campylobacter* sp. or *Yersinia* sp.

**Discussion**

A total of 2,000 flies from Southern Nigeria were screened for the intestinal colonization with antimicrobial resistant and enteropathogenic bacteria. Our main findings are a high proportion of *S. aureus* (13.8%, 275/2,000) and low occurrence of ESBL-E (0.8%, 16/2,000).

The analysis of the intestinal culturome was done to assess the occurrence of bacterial species independent of the resistance phenotype. In general, *Enterobacterales* were only rarely reported (e.g. *K. pneumoniae*, n = 1/82,
which is in line with an overall low colonization rate with ESBL-E in flies (0.8%). The vast majority of isolates belonged to the genera *Bacillus* and *Clostridioides* suggesting a selection of spore-forming bacteria by the treatment with ethanol. However, we confirmed the observation that ethanol sanitation of the exoskeleton does not alter the intestinal colonization\(^1\). Although the culturome was assessed under aerobic conditions, some *Clostridioides* species were detected. This applies for those species, that are known to be aerotolerant (i.e. *C. histolyticum*, *C. terium*, Fig. S1)\(^11\).

The *S. aureus* intestinal colonization rate in our study (13.8%) is in stark contrast to a 0.4% colonization rate in flies in a comparable approach (i.e. same trap and bait) from Germany\(^12\). The differing proportions are most likely due to the different settings (tropical vs. temperate regions). All *S. aureus* from our study shared a very similar genetic background based on the *spa* repeat pattern, ST and cgMLST (Fig. 2). This is suggestive either for a common source for all isolates from flies or a cross-contamination between flies during sampling or an adaptation (due to e.g. fitness factors) of this ST15 lineage to the intestinal tract of the flies. An artificial contamination of flies would challenge the scientific significance of our work. However, the widespread detection of *S. aureus* from various sampling site (Fig. 1), both the absence and presence of *mecA* in isolates belonging to t674, different antimicrobial resistance rates, the high diversity of ESBL-E sampled simultaneously and the known effective sanitation of the exoskeleton by ethanol argues against a cross-contamination during sampling\(^13\). All isolates belonged to ST15, which is in line with a predominance of the clonal complex CC15 in isolates from community-acquired infection in sub-Saharan Africa\(^14\). In addition, CC15 is known to be well adapted to the human host\(^15\). However, the low antimicrobial resistance rates, the absence of *lukF*-PV/*lukS*-PV and *sak* might also suggest that *S. aureus* from flies are rather of animal (e.g. livestock or wildlife) than of human origin\(^16–18\). However, other factors not investigated in our study (e.g. adaptation of *S. aureus* to the gut of ‘filth flies’, fitness factors) could explain the predominance of ST15/t674-*S. aureus* in flies.

Among all *S. aureus* from ‘filth flies’, we only detected *sea* and *sei*. The superantigenic activity of both is superior to other staphylococcal enterotoxins (e.g. members of the SEB group) due to an additional high-affinity MHC II binding site\(^19\).

The intestinal colonization rate of ‘filth flies’ with ESBL-E was low (0.8%, 16/2,000) compared to a similar study from Germany (3.3%, 44/1346)\(^12\). This finding is surprising as ESBL-E rates are high both in asymptomatic carriers (33.6%, rectal) and among *Enterobacterales* from bloodstream infection (12.1–15%) in sub-Saharan Africa\(^20–22\).

Although our study has strengths (e.g. large sample size, broad bacterial culture), some limitations need to be addressed: first, culturome results revealed the abundance of *Bacillus* and *Clostridioides* suggesting a selection of spore-forming genera by ethanol. This might point towards a methodological bias of our work. However, others have also reported a higher prevalence of *Bacillus cereus* than *Enterobacteriaceae*\(^23\). Second, despite poor sanitation systems and access of flies to human and animal faeces, we did not detect any other enteropathogens (e.g. Figure S1) which is in line with an overall low colonization rate with ESBL-E in flies (0.8%). The vast majority of isolates belonged to the genera *Bacillus* and *Clostridioides* suggesting a selection of spore-forming bacteria by the treatment with ethanol. However, we confirmed the observation that ethanol sanitation of the exoskeleton does not alter the intestinal colonization\(^1\). Although the culturome was assessed under aerobic conditions, some *Clostridioides* species were detected. This applies for those species, that are known to be aerotolerant (i.e. *C. histolyticum*, *C. terium*, Fig. S1)\(^11\).

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Salmonella sp., Shigella sp.). Since we were unable to culture the fly samples immediately, some isolates might have not stayed viable during storage and transport.

In conclusion, diptera-borne S. aureus food poisoning might be or become a health issue in the study region due to the high prevalence of enterotoxins (sea, sei) in S. aureus from ‘filth flies’. In contrast, a transmission of ESBL-E through flies by defecation and regurgitation does not seem to play a major role.

Materials and methods

Ethical statement. An ethical approval is not required for the analysis of invertebrates. All methods were carried out in accordance with relevant guidelines and regulations. The local Ph.D. committee, Medical Faculty, Westfälische Wilhelms-Universität Münster, approved all experimental protocols.

Study area/mapping. ‘Filth flies’ were collected in Southern Nigeria between June and July 2017. Sampling sites were classified into “urban”, “semi-urban” and “rural” according to the European Union Methodological manual on territorial typologies24. GPS coordinates were taken for each sampling site (eTrex 10, Garmin, Olathe, Kansas, USA). For every sampling spot, key environmental conditions were documented (i.e. livestock/human faeces within 10 m radius, presence of refuse dump, presence of decomposing organic matters and setting [urban, semi-urban, rural]). Atmospheric conditions (i.e. humidity, temperature, air pressure, wind force, sunshine hours) were recorded as reported by the Nigerian Meteorological Agency (NiMet, https://nimet.gov.ng/).

The maps were downloaded as jpg-files from openstreetmap (https://www.openstreetmap.org) with bounding boxes (latitude, longitude) related to the region of interest and used as a background for the plots. All plots were produced with “R” (Version 3.6.2) using the package “ggplot2” (grammar of graphics, version 3.2.0)25. The data used for the plots were taken from the original files and, where necessary, transformed by methods from the package “dplyr” (grammar of data manipulation, version 0.8.3).

Diptera. Flies (n = 2,000) were collected using the Gaze trap method25. Insect bait, conventionally made from animal proteins, carbohydrates and sugar (Feldner, Waldsee, Germany) in a container covered with gauze and placed under the gaze trap was used to lure the flies. Trapped flies (approximately 20/sampling site) were collected and killed in 70% ethanol and dried in silica gel (2–4 mm, Carl Roth, Karlsruhe, Germany). Ethanol sanitizes the outer surfaces of the flies, avoiding cross-contamination during sampling without altering the intestinal microbiome of the flies26.

Each fly was sent for further analysis to Germany in 1 ml sterile sodium chloride (0.45%) at -18 °C (Peli Biothermal Credo Cube, UK).

Culturome of random flies. We analysed the culturome of 82 randomly selected flies in order to describe the overall colonization pattern of flies with bacteria independent of the AMR phenotype. Since our target bacterial species (e.g. Escherichia coli, Klebsiella sp.) are aerobic bacteria, we did not use specific anaerobic conditions for culture. After removal of the legs and wings for molecular species identification, the remaining body (head, thorax and abdomen) was mechanically homogenized and cultured in 1 ml BHI broth overnight (37 °C, ambient air). A total of 10 µl of overnight culture was sub-cultured on MacConkey Agar (Oxoid GmbH, Wesel, Deutschland), Columbia Blood Agar (Oxoid), Trypticase Soy Agar (BD, Heidelberg, Germany), Kimmig Agar (Oxoid), Chocolate Agar (BD) and Colistin-Aztreonam (CAP) Agar (Oxoid). All phenotypically different colo-

ESBL-E and enteropathogenic bacteria. After mechanical homogenization, flies (i.e. head, thorax and abdomen) were cultured in 1 ml BHI overnight (37 °C, ambient air). Of this overnight culture, 10 µl were sub-cultured each on Columbia Blood Agar (for growth control) and selective media for the detection of S. aureus (SAID, bioMérieux, Marcy l’Etoile, France), ESBL-E (chromID, bioMérieux), Shigella sp. and Salmonella sp. (S agar, Oxoid), Campylobacter sp. (Campylobacter, Oxoid) and Yersinia sp. (CIN, Oxoid). For Salmonella sp., 10 µl of the overnight culture was additionally transferred to Müller-Kauffmann Tetradionate-Novobiocin broth (Oxoid) for a second overnight enrichment step and then sub-cultured on Onöz Agar (Oxoid).

Identification and characterization. Species of suspected S. aureus was identified using MALDI-TOF and confirmed by the detection of a species specific polymorphism of the non-ribosomal peptide synthetase (NRPS)28 and the S. aureus specific thermostable nuclease (nuc)29. All S. aureus were screened for the immune evasion cluster (IEC)29, the Panton-Valentine leucocidin gene ( lukS-PV/lukF-PV)30 and the enterotoxin genes sea, seb, sec, sed, see, sef, seg, seh and sei29,31.

Enterobacteriales were identified with VITEK2 automated systems (bioMérieux) due to ambiguous delineation of E. coli and Shigella sp. using MALDI-TOF.

Antimicrobial resistance. The antimicrobial susceptibility testing was done with VITEK2 automated systems (bioMérieux) using EUCAST clinical breakpoints (Version 9.0). ESBL-E were confirmed using the double disc diffusion test (Mast diagnostics, Bootle, UK) and were screened for the presence of blashv, blacTX-M, blatem

and blacCMY-232,33. Subtypes of the detected beta-lactamase genes were determined by Sanger sequencing.

All S. aureus isolates were screened for the staphylococcal beta-lactamase blaZ.34.
Genotyping. All *S. aureus* were spa typed and one isolate per spa type was selected for multilocus sequence typing (MLST)\(^3,36\). Using an online randomization tool (www.random.org), 13 *S. aureus* isolates were randomly selected for whole genome sequencing (WGS). This was done in order to understand the extent of diversity between the isolates on a whole genome level.

ESBL-producing *E. coli* were phylotyped\(^37\). Non-phylotypable strains were subjected to WGS to deduce the MLST sequence type (ST).

Whole genome sequencing. Genomes of selected isolates of ESBL-E and *S. aureus* were sequenced on an Illumina MiSeq platform (Illumina Inc., San Diego, USA) with a ≥ 75-fold coverage. Quality trimming and de novo assembly using the Velvet assembler was done with SeqSphere+ (version 5.9.0; Ridom GmbH, Münster, Germany)\(^38\). Neighbor-joining trees were constructed based on the cgMLST scheme to assess the clonal relation of the tested isolates\(^39,40\).

Fly species identification. All flies being colonized with the target organisms (*n* = 291) plus 109 randomly selected non-colonized flies from each of the sampling spots were selected for species identification. Legs and wings of flies were crushed using a sterile pestle for DNA extraction (QIAamp DNA Mini Kit, Qiagen, Hilden, Germany). The washing and DNA purification were done according to the manufacturer’s instruction. After amplification, the cytochrome oxidase gene (*coi*) was Sanger sequenced and query sequences were aligned with sequences deposited at the BLAST database for nucleotides\(^41\). The best match (≥ 99% coverage, ≥ 99% identity) was selected for species identification.

Statistical analysis. Proportions of categorical variables (e.g. colonization with ESBL-E, *S. aureus*) were compared using the Pearson’s chi-squared test or Fisher’s exact test where appropriate and normally distributed continuous variables were compared with student’s t-test. All environmental factors potentially associated with the detection of *S. aureus* in flies (*p* < 0.25) were entered in a multivariate logistic regression model with a stepwise backward elimination\(^37\). The significance level was set at 0.05. Analyses were done using “R”\(^25\).

The datasets generated during and/or analysed during the current study as well as all bacterial strains are available from the corresponding author on reasonable request.

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**Author contributions**

F.S. conceived and supervised the project and analysed data. F.O. performed the experiments and analysed data. A.M. performed and analysed whole genome sequencing. V.O.N. hosted the study in Nigeria. B.S. performed the geospatial analyses. E.O. and F.S. drafted the manuscript. All authors reviewed the manuscript and approved the final version.

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