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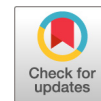
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Liquid Chromatography Mass Spectrometry Detection of Antibiotic Agents in Sputum from Persons with Cystic Fibrosis

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ABSTRACT Antibiotic therapy is expected to impact host microbial communities considerably, yet many studies focused on microbiome and health are often confounded by limited information about antibiotic exposure. Given that antibiotics have diverse pharmacokinetic and antimicrobial properties, investigating the type and concentration of these agents in specific host specimens would provide much needed insight into their impact on the microbes therein. Here, we developed liquid chromatography mass spectrometry (LC-MS) methods to detect 18 antibiotic agents in sputum from persons with cystic fibrosis. Antibiotic spike-in control samples were used to compare three liquid extraction methods on the Waters Acquity Quattro Premier XE. Extraction with dithiothreitol captured the most antibiotics and was used to detect antibiotics in sputum samples from 11 people with cystic fibrosis, with results being compared to the individuals' self-reported antibiotic use. For the sputum samples, two LC-MS assays were used; the Quattro Premier detected nanomolar or micromolar concentrations of 16 antibiotics, whereas the Xevo TQ-XS detected all 18 antibiotics, most at subnanomolar levels. In 45% of tested sputum samples (71/158), at least one antibiotic that was not reported by the subject was detected by both LC-MS methods, a discordance largely explained by the thrice weekly administration and long half-life of azithromycin. For ~37% of samples, antibiotics reported as taken by the individual were not detected by either instrument. Our results provide an approach for detecting a variety of antibiotics at the site of infection, thereby providing a means to include antibiotic usage data into microbiome studies.

KEYWORDS LC-MS, antibiotic, cystic fibrosis, sputum

Antibiotic usage is expected to alter host microbial composition in the treatment of infectious diseases (1, 2). It is, however, challenging to account for the impact of antibiotics on microbial community composition during the course of therapy without determining which antibiotics microbes encounter at the site of infection or elsewhere in the host. Obtaining reliable information to account for antibiotic use is particularly challenging in persons with chronic infections where antibiotic therapy is often intermittent and adherence to treatment recommendations is uncertain (3, 4). The levels of antibiotics at the actual infection-site are often unknown, as most pharmacokinetic studies measure antibiotics in serum (see Table S1 in the supplemental material) (5–36) with fewer assessing antibiotic levels at the infection site (10, 13, 15, 17, 27–32, 34–40).

Insofar as bacterial survival and gene expression are affected by antibiotic type and the local antibiotic concentration (41), there is a need for objective methods to account

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TABLE 1 Multiple reaction monitoring parameters for the Acquity Quattro Premier XE and Xevo TQ-XS

Analyte ^a	Quattro Premier XE TQ ^b				Xevo TQ-XS	
	MS/MS	CV (V)	CE (V)	RT (min)	MS/MS:	RT (min)
Amikacin	585.98 > 163.36	30	30	0.39	585.1 > 163	0.19
Amoxicillin	365.98 > 349.25	20	10	1.08	366.1 > 208	1.05
Ampicillin	350.00 > 106.36	20	20	3.14	350.1 > 106	1.34
Azithromycin	749.25 > 591.75	50	30	3.44	749.5 > 591.2	1.57
Aztreonam	435.94 > 313.27	20	20	2.61	436.1 > 313	1.23
Cefepime	480.99 > 123.35	20	50	0.81	481.1 > 167	1.01
Ceftazidime	546.91 > 468.22	30	20	2.08	547.1 > 396	1.19
Ceftriaxone	554.85 > 167.39	20	30	2.95	555.1 > 167	1.31
Ciprofloxacin	332.08 > 231.25	30	40	3.04	332.1 > 288	1.37
Colistin	1,155.41 > 729.34*	80	40	2.1	587.72 > 456.4	1.4
Levofloxacin	362.07 > 318.33	30	20	2.93	362.1 > 318	1.35
Linezolid	338.10 > 296.29	40	20	3.34	338.1 > 296	1.65
Meropenem	384.07 > 68.54	30	40	2.34	384.1 > 141	1.22
Piperacillin	518.04 > 143.37	30	20	3.46	518.1 > 160	1.93
Sulfamethoxazole	254.03 > 92.39	30	30	3.04	254.1 > 156	1.6
Tobramycin	467.96 > 167.22	40	20	2.08	468.1 > 167	1.19
Trimethoprim	291.11 > 230.24	40	20	2.89	291.1 > 230	1.34
Vancomycin	448.38 > 1305.91*	40	20	1.63	725.63 > 1307.23	1.17
Levofloxacin-d ₈	371.10 > 326.38	40	20	2.93	370.1 > 326.1	1.35
Linezolid-d ₃	341.10 > 297.29	40	20	3.34	341.1 > 297.1	1.65

^aLevofloxacin-d₈ and linezolid-d₃ are the internal standards.

^bCV, cone voltage; CE, collision energy; RT, retention time; *, low response from the M + H ions for vancomycin and colistin on the Quattro Premier.

for antibiotics in assessing the dynamics of microbial communities in infectious diseases. To detect antibiotics in clinical samples, we developed two low-cost, high-throughput ultraperformance liquid chromatography-tandem mass spectrometry (UPLC-MS/MS) methods. We investigated the utility of these methods by examining sputum samples from persons with cystic fibrosis (CF), a condition where incomplete antibiotic use data contributes to confounding assessment of treatment outcomes (1, 42–50). Individuals with CF experience chronic polymicrobial airway infections (1, 46–48, 50–56), and intensive antibiotic use is often poorly documented in the medical record. We assessed our results in the context of self-reporting of antibiotic use by these individuals.

RESULTS

Detection of antibiotics on the Waters Quattro Premier XE and Xevo TQ-XS UPLC-MS/MS. We compared two UPLC-MS/MS instruments for their ability to separate and quantitate 18 antibiotics commonly prescribed to individuals with CF (Table 1). Run conditions, including comparison of mobile phases (see Text S1 and Table S2 in the supplemental material), were first optimized on the Waters Quattro Premier XE UPLC-MS/MS at the University of California, Irvine Mass Spectrometry Facility. A mobile phase consisting of a water-methanol gradient with 2 mM ammonium acetate and 0.1% acetic acid (57) chromatographically separated 16 of 18 antibiotics with peak areas at least 100 times higher than background (Fig. 1). However, neither vancomycin nor colistin were consistently detected with either mobile phase solvent on the Quattro Premier due to low response from the protonated molecular ion (M + H). The lower limits of detection (LODs) of the external standards ranged from 0.001 mg/liter (meropenem) to 16.5 mg/liter (cefepime) (see Table S3A in the supplemental material).

Antibiotic standards were also optimized on the Xevo TQ-XS at the Waters Corporation Demo Laboratory (Beverly, MA). Colistin and vancomycin parameters were manually optimized on the Xevo by scanning and identifying multiple protonated forms (M + 2H). While the Xevo parameters were different from the Quattro Premier and comparisons in the LODs between the two instruments are imperfect, the Xevo LODs were on average 10,000-fold lower than the Quattro Premier (Fig. 2). The Xevo LODs ranged from 0.002 μg/liter (sulfamethoxazole) to 5.8 μg/liter (colistin) (Table

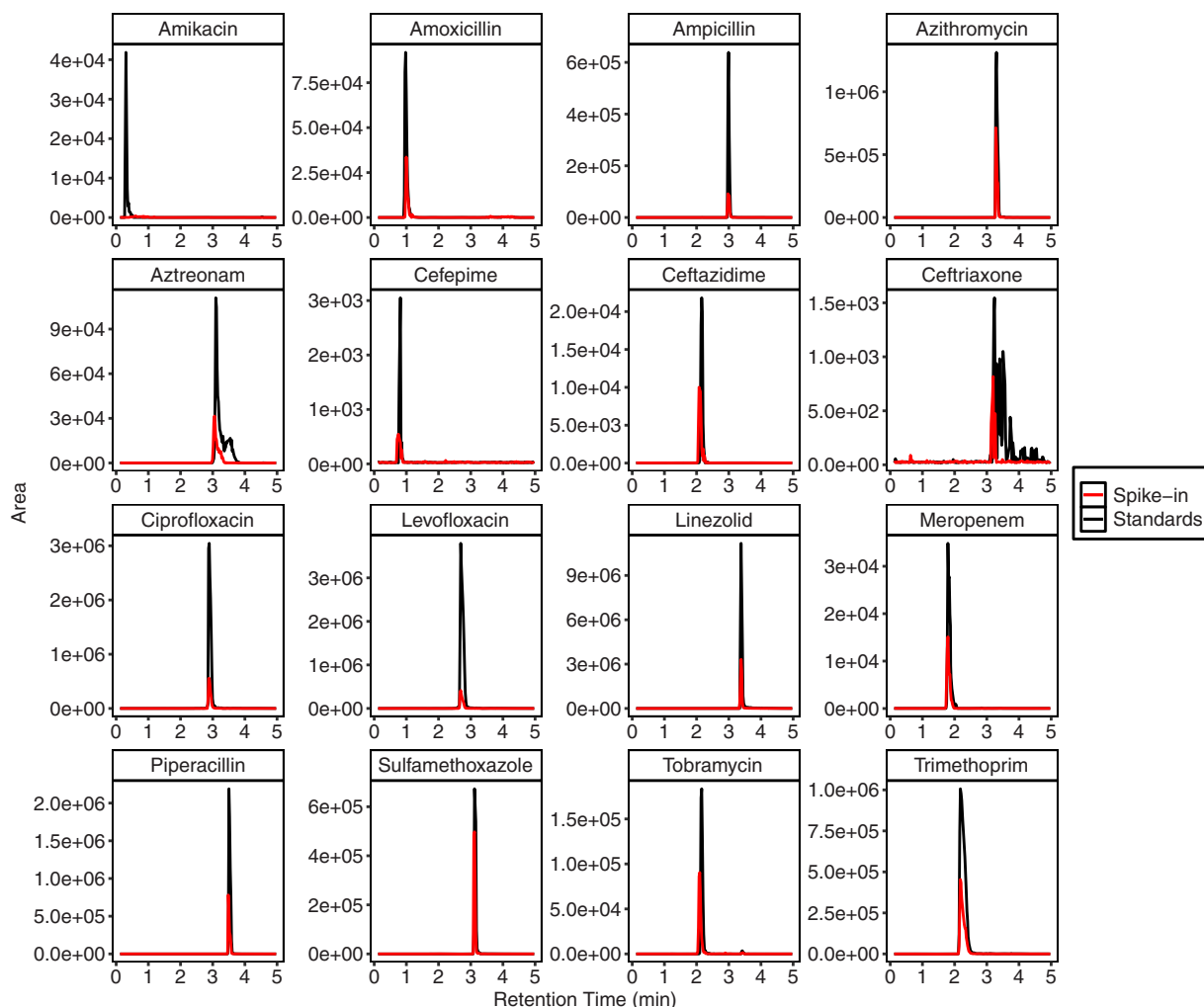


FIG 1 Quattro Premier XE extracted ion chromatograms (XIC) of each antibiotic from the 10 μ M pool of external standards (black line) and from the artificial sputum medium spike-in experiments (red line) using water-methanol gradient plus 2 mM ammonium acetate and 0.1% acetic acid mobile phase method. XICs are ordered by retention time. No Quattro Premier XICs are shown for vancomycin and colistin due to inconsistent detection.

S3A). Multiple reaction monitoring (MRM) parameters for both instruments are listed in Table 1.

Comparison of antibiotic extraction protocols in artificial sputum medium for detection on the Quattro Premier. We compared three extraction solvents for efficiency in recovering antibiotics from sputum. Artificial sputum medium (ASM) was used as the matrix because antibiotic-free sputum was unavailable (Text S1). Most individuals who expectorate sputum regularly take antibiotics. For most of the antibiotics, recovery accuracy, precision, and LODs were similar across all three extraction solvents (see Text S1 and Table S3B to C), the exceptions being meropenem, ceftriaxone, and amoxicillin, which had lower LODs in 1% dithiothreitol (DTT) than MeOH or acetonitrile/acetic acid (57) (Text S1 and Table S3B).

Recovery of antibiotics from cystic fibrosis sputum and reproducibility. Because antibiotic-free sputum was not available for matrix-matched calibration (58), the concentrations of antibiotics detected in sputum were determined using a surrogate calibration approach (external standards dissolved in water and two internal standards spiked into samples [see Materials and Methods]). The accuracy of the surrogate calibration varied for each antibiotic (see Text S1 and Table S4 in the supplemental material). The mean relative error ranged from 15.4% for trimethoprim to 494% for

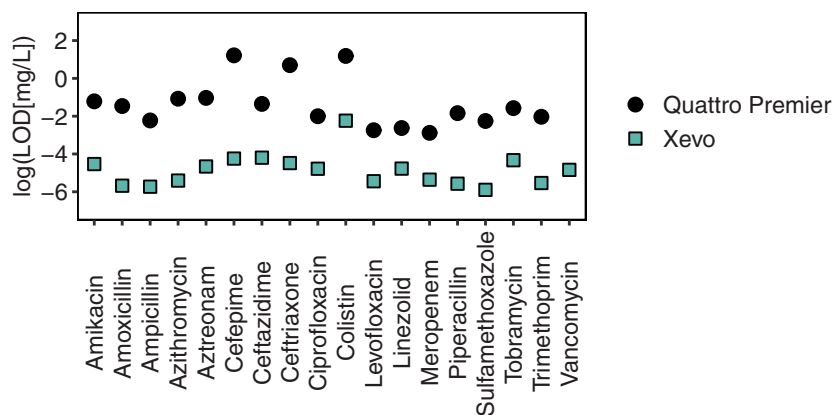


FIG 2 Comparison of the log-transformed limits of detection (LOD) for antibiotic standards on the Quattro Premier XE and the Xevo TQ-XS. On average, the Xevo TQ-XS LOD was 9,500-fold lower than that of the Quattro Premier.

linezolid (Table S4). For most of the antibiotics, the measured concentration was higher than the added concentration even after background subtraction (Text S1 and Table S4), and the relative error was higher in the sputum spike-in experiments relative to that of the ASM experiments (Table S3C).

Antibiotic extraction and chromatography conditions were initially tested on three replicate aliquots from each of three CF sputum samples on the Quattro Premier (see Fig. S1B and Text S1 in the supplemental material). The Quattro Premier method reproducibly detected two out of four of the antibiotics taken by the subject, azithromycin and trimethoprim (coefficient of variation [COV] < 30%). The detected concentration of tobramycin was near the liquid chromatography mass spectrometry (LC-MS) detection limits (Fig. S1B and Text S1).

Detection of antibiotics in clinical study sputum samples. The optimized LC-MS antibiotic assays were then tested on 171 sputum samples from 11 subjects with CF. Antibiotic use was reported by subjects on the same day as sample collection for 158 of the sputum samples. Subjects took 11 of 18 antibiotics detected by the LC-MS assays by oral, inhaled, or intravenous (i.v.) routes (azithromycin, aztreonam, ceftazidime, ciprofloxacin, colistin, levofloxacin, linezolid, trimethoprim-sulfamethoxazole, tobramycin, and vancomycin) (Fig. 3 and Table 2; see also Fig. S2 in the supplemental material). Oral azithromycin was the most common antibiotic reported as taken (10 subjects, 75 samples), followed by inhaled aztreonam (8 subjects, 53 samples) and oral ciprofloxacin (5 subjects, 3 samples) (Fig. 3). The most common combination taken on the same day was oral azithromycin and inhaled aztreonam (7 subjects, 22 samples) (Fig. S2). Of the remaining seven antibiotics in the LC-MS assay, ampicillin and ceftriaxone were not a survey option but were included because these antibiotics could be prescribed to individuals with CF.

To reduce technical variability in our extraction method, all 171 samples were processed and run in one batch (total run time of 23.5 h, including washes). As a technical quality control (QC), a pool of the 18 antibiotics was run every 57 samples. The intensity of the QC pools declined 50% on average by the end of the Quattro Premier run (see Fig. S3B in the supplemental material), while the Xevo run did not have a drop in the internal standard response over time, likely due to lower injection volume and less protein input on the column (0.25 mg versus 5 mg per sample) (Fig. S3C).

The Quattro Premier and Xevo detected azithromycin, aztreonam, ceftazidime, ciprofloxacin, levofloxacin, linezolid, sulfamethoxazole, tobramycin, and trimethoprim in CF sputum samples. The Xevo alone detected colistin and vancomycin, and the Quattro Premier alone detected ampicillin, ceftriaxone, and piperacillin (Table 3). The concentrations of detected antibiotics were determined with the surrogate calibration approach (Fig. 4; see also Fig. S4 in the supplemental material). Although most of the

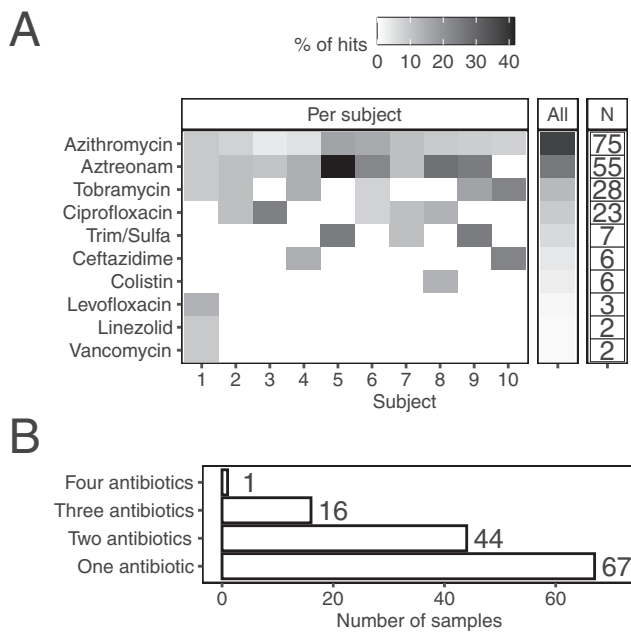


FIG 3 Daily self-reported antibiotic usage by 10 subjects with cystic fibrosis on the same day that sputum samples were collected. (A) Cells are shaded on the grayscale heat map based on number of times a subject reported taking the specified antibiotic divided by the total number of times a subject reported taking any antibiotics (“Per subject” columns) and across the entire cohort (“All” column). *n* is the number of times an antibiotic was reported as taken on the sampling day (207 total reports of antibiotic usage for 128 sputum samples). Trim, trimethoprim; Sulfa, sulfamethoxazole. Subject 11 reported taking none of the antibiotics in this study set. (B) The number of sampling days where a subject reported taking 1 to 4 antibiotics on the same day.

antibiotics had high relative error values determined from the sputum recovery experiments (Table S4), the concentrations between the Quattro Premier and Xevo could be compared since the same extracted material was used on both platforms. The Xevo detection of antibiotic concentrations was overall lower than that of the Quattro Premier, likely due to declining stability of the antibiotics while shipping the extracted material to the Xevo lab (Fig. 4). Specifically, azithromycin and ciprofloxacin had significantly lower concentrations when samples were run on the Xevo (paired Wil-

TABLE 2 Survey options for the 18 antibiotics optimized for LC-MS detection^a

Antibiotic	Class	Survey option ^b	Reported by subjects	Detected by LC-MS
Amikacin	Aminoglycoside	Amikacin inhaled		
Amoxicillin	β -lactam	Amoxicillin clavulanate oral		
Ampicillin	β -lactam	Not provided	Not provided	Yes
Azithromycin	Macrolide	Azithromycin i.v. or oral	Oral	Yes
Aztreonam	β -lactam	Aztreonam i.v. or inhaled	i.v., inhaled	Yes
Cefepime	Cephalosporin	Cefepime i.v.		
Ceftazidime	Cephalosporin	Ceftazidime i.v.	i.v.	Yes
Ceftriaxone	Cephalosporin	Not provided	Not provided	Yes
Ciprofloxacin	Fluoroquinolone	Ciprofloxacin i.v. or oral	Oral	Yes
Colistin	Polymyxin	Colistin inhaled or i.v.	Inhaled	Yes
Levofloxacin	Fluoroquinolone	Levofloxacin oral	Oral	Yes
Linezolid	Oxazolidinone	Linezolid oral	Oral	Yes
Meropenem	β -lactam	Meropenem inhaled		
Piperacillin	β -lactam	Piperacillin i.v. or piperacillin tazobactam i.v.		Yes
Sulfamethoxazole	Sulfonamide	Trim/sulfa oral	Oral	Yes
Tobramycin	Aminoglycoside	Tobramycin i.v. or inhaled	i.v., inhaled	Yes
Trimethoprim	Trimethoprim	Trim/sulfa oral	Oral	Yes
Vancomycin	Glycopeptide	Vancomycin inhaled or i.v.	i.v.	Yes

^aAmpicillin and ceftriaxone were not provided as an option on the survey. Out of the 16 remaining antibiotics, 11 antibiotics were reported as taken. Fourteen antibiotics were detected in sputum on one or both of the LC-MS platforms.

^bTrim, trimethoprim; Sulfa, sulfamethoxazole.

TABLE 3 Contingency tables comparing LC-MS data to subject self-reported surveys for each antibiotic reported as taken by at least one subject^a

<p>Azithromycin</p> <p>QUATTRO:</p> <table border="1"> <tr><td colspan="2"></td><td colspan="2">SURVEY:</td><td>Sens=92%</td></tr> <tr><td colspan="2"></td><td>Yes</td><td>No</td><td>Spec=16%</td></tr> <tr><td>Yes</td><td>69</td><td>70</td><td></td><td>PPV=50%</td></tr> <tr><td>No</td><td>6</td><td>13</td><td></td><td>NPV=68%</td></tr> </table> <p>XEVO:</p> <table border="1"> <tr><td colspan="2"></td><td colspan="2">SURVEY:</td><td>Sens=99%</td></tr> <tr><td colspan="2"></td><td>Yes</td><td>No</td><td>Spec=13%</td></tr> <tr><td>Yes</td><td>74</td><td>72</td><td></td><td>PPV=51%</td></tr> <tr><td>No</td><td>1</td><td>11</td><td></td><td>NPV=92%</td></tr> </table>			SURVEY:		Sens=92%			Yes	No	Spec=16%	Yes	69	70		PPV=50%	No	6	13		NPV=68%			SURVEY:		Sens=99%			Yes	No	Spec=13%	Yes	74	72		PPV=51%	No	1	11		NPV=92%	<p>Aztreonam (Inhaled)</p> <p>QUATTRO:</p> <table border="1"> <tr><td colspan="2"></td><td colspan="2">SURVEY:</td><td>Sens=36%</td></tr> <tr><td colspan="2"></td><td>Yes</td><td>No</td><td>Spec=100%</td></tr> <tr><td>Yes</td><td>19</td><td>0</td><td></td><td>PPV=100%</td></tr> <tr><td>No</td><td>34</td><td>97</td><td></td><td>NPV=74%</td></tr> </table> <p>XEVO:</p> <table border="1"> <tr><td colspan="2"></td><td colspan="2">SURVEY:</td><td>Sens=19%</td></tr> <tr><td colspan="2"></td><td>Yes</td><td>No</td><td>Spec=100%</td></tr> <tr><td>Yes</td><td>10</td><td>0</td><td></td><td>PPV=100%</td></tr> <tr><td>No</td><td>43</td><td>97</td><td></td><td>NPV=69%</td></tr> </table>			SURVEY:		Sens=36%			Yes	No	Spec=100%	Yes	19	0		PPV=100%	No	34	97		NPV=74%			SURVEY:		Sens=19%			Yes	No	Spec=100%	Yes	10	0		PPV=100%	No	43	97		NPV=69%	<p>Aztreonam (IV)</p> <p>QUATTRO:</p> <table border="1"> <tr><td colspan="2"></td><td colspan="2">SURVEY:</td><td>Sens=100%</td></tr> <tr><td colspan="2"></td><td>Yes</td><td>No</td><td>Spec=100%</td></tr> <tr><td>Yes</td><td>2</td><td>0</td><td></td><td>PPV=100%</td></tr> <tr><td>No</td><td>0</td><td>6</td><td></td><td>NPV=100%</td></tr> </table> <p>XEVO:</p> <table border="1"> <tr><td colspan="2"></td><td colspan="2">SURVEY:</td><td>Sens=100%</td></tr> <tr><td colspan="2"></td><td>Yes</td><td>No</td><td>Spec=100%</td></tr> <tr><td>Yes</td><td>2</td><td>0</td><td></td><td>PPV=100%</td></tr> <tr><td>No</td><td>0</td><td>6</td><td></td><td>NPV=100%</td></tr> </table>			SURVEY:		Sens=100%			Yes	No	Spec=100%	Yes	2	0		PPV=100%	No	0	6		NPV=100%			SURVEY:		Sens=100%			Yes	No	Spec=100%	Yes	2	0		PPV=100%	No	0	6		NPV=100%
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^aAztreonam and tobramycin were reported as taken inhaled or intravenously (i.v.). Sens, sensitivity; spec, specificity; PPV, positive predictive value; NPV, negative predictive value.

coxon rank sum test, $P < 0.05$; azithromycin Quattro Premier mean = 227 mg/liter, azithromycin Xevo mean = 34 mg/liter; ciprofloxacin Quattro Premier mean = 2.1 mg/liter, ciprofloxacin Xevo mean = 1.6 mg/liter). In addition, the Quattro Premier method detected aztreonam and tobramycin in more samples than the Xevo ($n = 9, 2$). Antibiotics in several samples were detected only by the Xevo, including azithromycin ($n = 2$), ceftazidime ($n = 1$), and trimethoprim ($n = 1$). In addition, the Xevo detected colistin ($n = 1$) and vancomycin ($n = 1$), antibiotics that were not measured with the Quattro Premier method (Tables 3; see also Table S4).

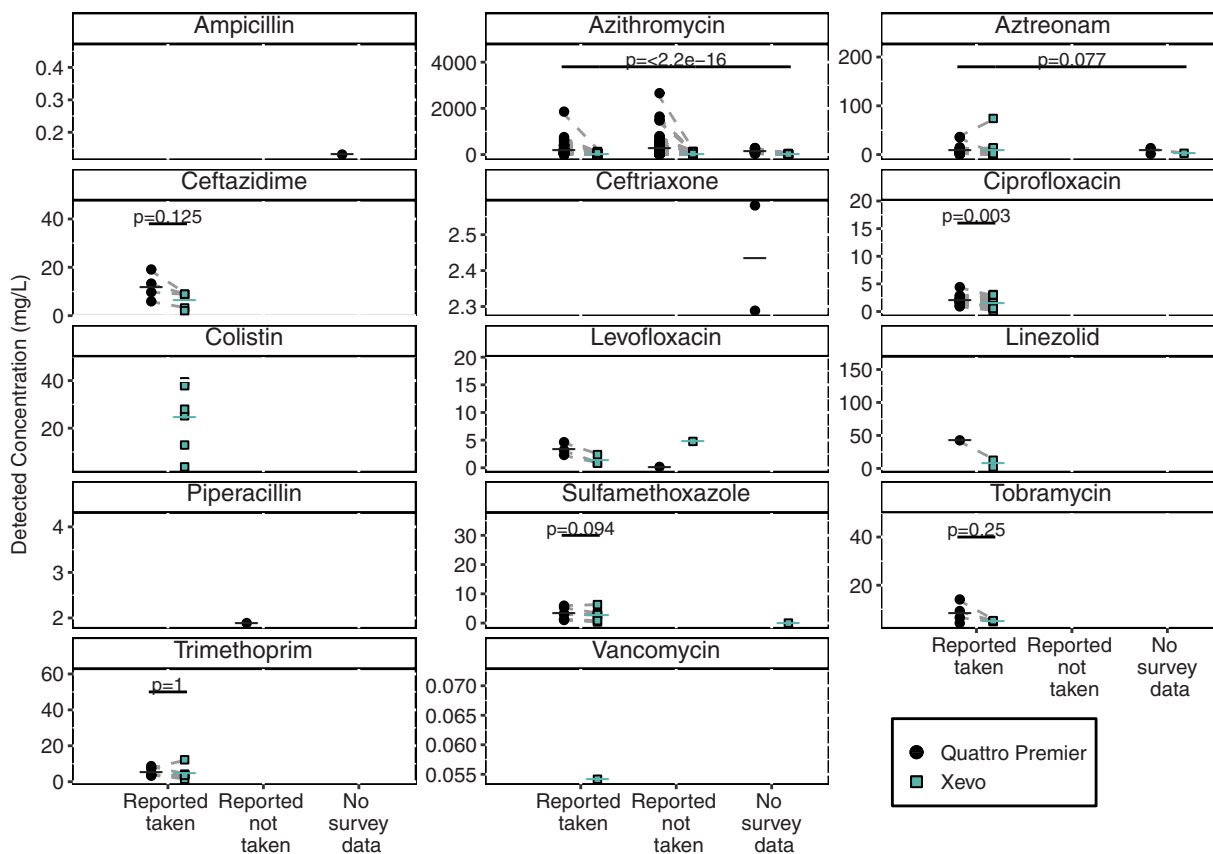


FIG 4 Concentration (mg/liter) of detected antibiotics reported as taken or not taken by a subject in 158 sputum samples. Subjects did not provide any antibiotics usage data for 13 samples. Ampicillin and ceftriaxone were not included as an option in the antibiotic usage survey. Points are concentrations of the individual samples for the Quattro Premier (black circle) and the Xevo (turquoise square). Samples containing antibiotics detected by both instruments are connected by a dashed gray line. A paired Wilcoxon rank sum test was used to determine if the Quattro Premier and Xevo detected concentrations were significantly different for antibiotics that had at least three paired samples (azithromycin, aztreonam, ceftazidime, ciprofloxacin, sulfamethoxazole, tobramycin, trimethoprim). Azithromycin and ciprofloxacin had significantly different mean detected concentrations detected by the two instruments (paired Wilcoxon rank sum test, $P < 0.05$; azithromycin Quattro Premier mean = 227 mg/liter; azithromycin Xevo mean = 34 mg/liter; ciprofloxacin Quattro Premier mean = 2.1 mg/liter; ciprofloxacin Xevo mean = 1.6 mg/liter).

LC-MS concordance with subject self-reported usage. To assess the performance of the LC-MS platforms, we compared the results of the sputum assays with source subjects' self-reported antibiotic usage. For each antibiotic, we calculated sensitivity, specificity, positive predictive value, and negative predictive value (using self-report as the gold standard) for both platforms (Table 3). Among the 10 antibiotic treatments reported as taken by the subjects, we analyzed trimethoprim and sulfamethoxazole separately in addition to inhaled and i.v. tobramycin and aztreonam.

For both the Quattro Premier and Xevo, inhaled tobramycin had the lowest sensitivity (detected in 0/20 samples). Intravenous aztreonam and oral levofloxacin had the highest sensitivity (100%), but the sensitivity is likely inflated due to the low number of samples (Table 3). The Quattro Premier and Xevo had similar sensitivities for azithromycin, ciprofloxacin, levofloxacin, sulfamethoxazole, i.v. tobramycin, and trimethoprim. The Xevo was able to detect colistin (1/2) and vancomycin (6/6), whereas both of these antibiotics were not detectable with the Quattro Premier method. The Xevo also had higher sensitivity rates than the Quattro Premier for ceftazidime and linezolid, although both of these antibiotics were reported in only six and two samples, respectively. In contrast, the Quattro Premier had higher sensitivity for i.v. aztreonam than the Xevo. The Quattro Premier and Xevo specificities for nine of the antibiotics were 100%, the exception being azithromycin, which had specificities of 16% and 13%, respectively.

Detection of antibiotics on days without self-reported usage. The Quattro Premier and Xevo both detected antibiotics reported as not taken on the day of sample

collection. The percent of samples containing at least one unreported antibiotic was approximately 46% (72/158) by either LC-MS approach. The top antibiotics detected with LC-MS but reported as not taken on that specific sampling day were azithromycin (Quattro Premier $n = 70$; Xevo $n = 72$) and levofloxacin (Quattro Premier $n = 1$; Xevo $n = 1$; see Fig. S4A and C) (Fig. 4; Table 3). In all of the samples where azithromycin was detected, the subjects reported taking azithromycin in the last 8 days prior to sample collection (see Fig. S4A to E and H to J).

The Quattro Premier alone also detected piperacillin in one sample (Fig. 4 and Table 3; see also Fig. S4F), ceftriaxone in five sputum samples, and ampicillin in one sample (Fig. 4; see also Fig. S1B and S4A and H). Ceftriaxone and ampicillin were not included as an option on the antibiotic usage survey. Electronic medical records indicate that the subjects were not prescribed ceftriaxone or ampicillin around the time of sample collection and suggest that these antibiotics were false positives.

We wanted to determine if detection of unreported antibiotics correlated with subject symptom score, with the reasoning that subjects may take additional antibiotics during periods of worsening symptoms. However, samples with detected antibiotics did not correlate with symptom score (see Fig. S5A in the supplemental material) (Pearson correlation Quattro Premier, $df = 205$, $P = 0.44$, $r = 0.05$; Xevo, $df = 210$, $P = 0.85$, $r = -0.01$).

Impact of antibiotic half-life, storage condition, and route of delivery. The antibiotics with the lowest incidence of detection were inhaled tobramycin and inhaled aztreonam, which had sensitivities of 0 to 36% (Table 3). It is unlikely that instrument limitation reduced the detection rate for most of the antibiotics. The LODs from the Xevo did not significantly correlate with the agreement rate, and the Quattro Premier LODs were weakly, but not significantly, negatively correlated with agreement rate (see Fig. S5B) (Pearson's correlation Xevo, $df = 9$, $P = 0.47$, $r = 0.24$; Quattro Premier, $df = 7$, $P = 0.14$, $r = -0.53$). The one antibiotic potentially impacted by instrumentation limitation was tobramycin, which had the second worst detection limit for the Xevo ($0.05 \mu\text{g/liter}$) (Table S3A). In addition, instances of antibiotics being undetected by the LC-MS method were not due to the number of days sputum samples were stored at 4°C at subjects' homes (Fig. S5C) (Pearson's correlation Quattro Premier, $df = 284$, $P = 0.1$, $r = 0.1$; Xevo, $df = 285$, $P = 0.34$, $r = 0.06$).

Undetected antibiotics could be due to inadequate delivery to the airways or clearing of the antibiotic by the time of sampling, as subjects reported usage within a 24-h window of collection. In support of this, antibiotics with shorter half-lives, as determined from cystic fibrosis pharmacokinetic studies of serum (see Table S1 in the supplemental material), were less likely to be detected (Fig. S5D) (Pearson's correlation Quattro Premier, $df = 262$, $P < 0.05$, $r = -0.57$; Xevo, $df = 263$, $P < 0.05$, $r = -0.69$). Aztreonam was the second most undetected antibiotic and has a short half-life (9, 10) (Table 3).

Two of the 11 antibiotics were taken by subjects through inhaled or i.v. routes. The i.v.-administered tobramycin was detected more often by the LC-MS than inhaled tobramycin (Table 3). A similar trend was seen with aztreonam, although there were not enough i.v.-administered samples to confirm this statistically.

DISCUSSION

Although antibiotics are expected to be drivers for shaping the human microbiome, studies of human microbial ecology rarely account for the effect of antibiotics on changes in microbial community composition. An obstacle to a better understanding of the impact of antibiotics in this regard is the difficulty inherent in reliably ascertaining antibiotic usage, particularly in the context of prolonged or chronic therapy, and determining antibiotic presence in human tissues of interest. With respect to studies of the airway microbiome in persons with CF, antibiotic therapy is often not taken into account at all or is derived from prescribing information gleaned from the medical record (1, 42, 44–51), which is recognized as marginally reliable (3, 4). We, therefore, sought to investigate the utility of LC-MS to objectively determine the presence of

antibiotics, which could, in turn, be taken into account in analyses of microbial community dynamics in studies of the CF airway microbiome.

LC-MS performance and extraction efficiencies are antibiotic dependent. We explored the utility of two LC-MS methods and observed that these differed in terms of reliability in detecting the antibiotics included in our study. The Quattro Premier method detected levofloxacin and meropenem with the highest signals, while the Xevo assay performed best in the detection of ampicillin and sulfamethoxazole. Both instruments performed poorly in the detection of cefepime and ceftazidime, likely due to low binding of these hydrophilic cephalosporins to the reverse-phase column (see Table S3A in the supplemental material). The extraction efficiencies from ASM (represented by the apparent limit of detection [ALOD]) correlated with the LOD of the external standards. However, some of the antibiotics with low external standard detection limits had poor extraction efficiencies, including meropenem (see Table S3B and Fig. S1A in the supplemental material). This may have been due to the use of ASM, which contains major sputum components, including extracellular DNA, ferritin, chloride ions, sugars, and mucin sourced from porcine stomach. Our extraction protocol did not precipitate total protein content out of the sample, which could have reduced the extraction of antibiotics that interact with mucin or other proteins (59). Conversely, the use of ASM over sputum could have inflated extraction efficiency for certain antibiotics, since ASM does not contain immune cells. Azithromycin is known to accumulate in polymorphonuclear leukocytes, which likely impacts its delivery to the airways and decreases extraction efficiency from sputum (30).

Antibiotic concentrations in cystic fibrosis sputum. Because antibiotic-free sputum was not available for matrix-matched calibration, a surrogate calibration approach was used (58), antibiotic standards in solvent and internal standards spiked into the samples. Sputum recovery experiments indicated that the surrogate calibration approach can quantitate trimethoprim, azithromycin, aztreonam, levofloxacin, ciprofloxacin, and ampicillin with relative errors lower than 50% (see Table S4 in the supplemental material). Trimethoprim was the only antibiotic with a relative error of 15% (Table S4) (58).

The mean concentrations of the antibiotics detected in sputum were less than or similar to previously reported ranges in sputum from pharmacokinetic studies (Table S4) (10, 13, 15, 17, 27–32, 34, 36, 38, 39). The lower concentrations in our study could be due to differences in dosing, drug administration, subject demographics, or timing (subjects in this study provided sputum samples within a 24-h window of taking antibiotics). The concentrations of azithromycin detected in sputum (maximum = 2,700 mg/liter) were higher than reported values in a long-term use study (12 to 53 mg/liter) (30), which is likely due to concentrations above the upper limit of quantification (LOQ) (23.6 mg/liter) for this assay.

Persistence of antibiotics with long half-lives. We *a priori* hypothesized that antibiotics detected in sputum but reported as not taken would be associated with subject symptom scores because persons with CF may take nonprescribed antibiotics when experiencing worsening symptoms (4, 60). However, the LC-MS data did not support this hypothesis, as unreported antibiotics were not correlated with subject symptom scores (see Fig. S5A in the supplemental material). Oral antibiotics taken by subjects, including levofloxacin, trimethoprim-sulfamethoxazole, ciprofloxacin, and azithromycin, were detected in sputum around the time of reported usage except for a few examples. Indeed, discordance between self-reporting surveys and LC-MS data was primarily due to azithromycin detected in a sample collected 1 or 2 days after the subject reported taking oral azithromycin. The common dosage for azithromycin for people with CF is an oral tablet three times a week (61, 62). While most instances of discordance were due to persistence of azithromycin a couple of days after taking the antibiotic, some samples contained azithromycin 7 to 8 days after a subject last reported taking it. Azithromycin has the longest reported half-life of the 18 antibiotics (see Table S1 in the supplemental material) and has been reported to persist in CF

sputum days after administration (63). The persistence of azithromycin can be attributed to its high tissue penetration, accumulation in phagocytes, and lack of metabolism by the liver (30, 64).

Undetected antibiotics could reflect ineffective concentrations throughout the infection site. A high proportion of antibiotics, particularly inhaled aztreonam and tobramycin, were reported as taken by the subject but not detected by either method (Table 3). We first wanted to determine if sample storage conditions impacted the detection rate because the stability of antibiotics decreases (65), and the metabolite profiles in CF sputum change significantly from storage at 4°C (66). However, the number of days a sample was stored at 4°C was not correlated with lack of detection, suggesting adequate sample storage conditions (Fig. S5C). Instead, undetected antibiotics were inversely correlated with the antibiotic pharmacokinetic half-lives. In support of this, aztreonam and tobramycin are reported to have short half-lives of approximately 2 h in single-dose pharmacokinetic studies (9, 10, 24, 25, 32, 67, 68) (see Table S1 and Fig. S5D).

Inhaled tobramycin was not detected by either LC-MS method, even during periods of repeated usage. In contrast, i.v.-administered tobramycin was detected 50% of the time (Table 3). Inhaled tobramycin has been detected in serum at higher or similar concentrations in sputum (32).

The concentrations of inhaled antibiotics in sputum are impacted by lung absorption, elimination, and distribution (69). Sputum samples are not a global representation of the entire airway, and secretions from different physical locations in the lungs vary in metabolite, antibiotic, and microbial composition (70–72). The undetected antibiotics might also be explained by subject nonadherence, which was as high as 20% in one cohort of adult CF patients (60). Reported reasons for skipping antibiotics included forgetting or for social reasons (60). It is also possible that undetected antibiotics were degraded by the time of sputum collection in our study, as the enrolled subjects were asked to report antibiotics taken anytime on the same day of sample collection.

Discordance between LC-MS and usage data is not due to instrument limitation because the undetected antibiotics (tobramycin, aztreonam, and azithromycin) had submicromolar or subnanomolar limits of detection, thresholds that are still below subinhibitory concentrations reported to impact bacterial physiology (Table S3B). There is no exact threshold that determines if an antibiotic concentration impacts microbes *in vivo*, as antibiotic efficacy is impacted by environmental factors, such as protein binding. Although this study only measured total (bound and unbound) antibiotic levels, the amount of unbound antibiotics is likely lower in sputum than in serum (32). Tobramycin binds to extracellular DNA and protein in CF sputum (59), and the bioactive concentration of tobramycin is reported to be one-third of total concentration detected in sputum (68). The impact of physiologically relevant concentrations of antibiotics on the microbiome is poorly understood, and future efforts will determine how antibiotic levels measured in sputum drive changes in bacterial composition.

Study limitations and recommendations. The subjects in this study agreed to participate in the antibiotics survey, and their adherence to antibiotic usage and accurate completion of the self-reported antibiotic surveys is likely not representative of all individuals. In addition, the subjects provided survey responses within a 24-h window of expectorating. Antibiotics not detected by either LC-MS could have degraded by the time of sampling.

Certain antibiotics were taken more frequently than others, which likely impacts the LC-MS sensitivities and specificities. While we optimized the LC-MS method for 18 antibiotics, only 11 antibiotics were reported as taken by the subjects in this cohort. Notably, azithromycin was the most common antibiotic reported as taken in this study period. Oral azithromycin is typically taken by persons with CF three times a week, which contributed to the high discordance between the LC-MS data and daily self-reported usage.

The Quattro Premier and Xevo methods were optimized in different facilities and are imperfect comparisons for antibiotic LODs. The ASM antibiotic spike-in extraction experiments were only performed on the Quattro Premier, and we also have more information about the Quattro Premier parameters since it is our in-house instrument. We also reiterate that the Quattro Premier method was unable to detect vancomycin and colistin and could only detect cefepime and ceftazidime at high concentrations (see Table S3). However, the Xevo could detect all four of these antibiotics at nanomolar levels. In addition, the lower injection volume for the Xevo contributed to cleaner chromatography. Excluding these examples, the detection profiles of the Quattro Premier aligned with the Xevo for frequently taken antibiotics. While the LC-MS methods do not currently meet standards set forth by the FDA Bioanalytical Method Validation Guidance for Industry (58), the ultimate goal is to obtain accurate, quantitative information about antibiotics in the CF airways. Given that the Quattro Premier platform at the UC Irvine Mass Spec Facility is accessible to the authors, one future direction is to improve the sensitivity of this approach with improved chromatography, cleaner extraction methods, and improved calibration approaches. Calibration with addition of antibiotic standards to aliquots from the same sputum sample would account for the unique composition of each sample.

The data for antibiotic half-lives came from pharmacokinetic studies in serum (Table S1). While we acknowledge that the half-lives in serum are likely different than in sputum, there are fewer studies characterizing antibiotic pharmacokinetics in sputum.

Conclusion. We aimed to develop a high-throughput method that would allow for detection of antibiotics present at the infection site, such as sputum from the CF airways. Direct observations of antibiotics are needed to be related to microbial composition measures along with other clinical data. Incorporating antibiotic data into microbial community composition models is challenging in the context of CF due to many factors, including the lack of a standardized antibacterial treatment regimen, the impact of individual subject factors on antibiotic efficacy, and the diverse properties of CF antibiotics. Our LC-MS approach has inherent limitations but is the first step toward including objective antibiotic data in CF studies. Future endeavors will determine how the local presence of antibiotics impacts the microbial community with paired quantitative LC-MS and microbiome data.

MATERIALS AND METHODS

Chemicals. Pharmaceutical grade or high-performance liquid chromatography (HPLC) grade antibiotics were dissolved in water, methanol, or an acetonitrile/acetic acid solution to make 1 mM or 10 mM stocks (see Table S1 in the supplemental material). Stocks of the external standards were made directly before each run due to reported low stability of some antibiotics at -20°C and -80°C (57, 65). The antibiotics were then diluted with water and pooled to make a $10\ \mu\text{M}$ stock. For the external standard curve, a 3-fold dilution series was used as follows: $10\ \mu\text{M}$, $3.3\ \mu\text{M}$, $1.1\ \mu\text{M}$, $0.37\ \mu\text{M}$, $0.123\ \mu\text{M}$, $0.041\ \mu\text{M}$, and $0.014\ \mu\text{M}$. Internal standards linezolid- d_3 and levofloxacin- d_8 (Toronto Research Chemicals Inc., Ontario, Canada) were dissolved in water and methanol, respectively. Aliquots of the internal standards were stored at -80°C .

Quattro Premier XE optimization. Standards, optimization samples, and sputum samples were first run on the Quattro Premier XE UPLC-MS/MS (Waters Corp., Milford, MA) at the University of California, Irvine's Mass Spectrometry Facility. An Acquity UPLC ethylene-bridged hybrid (BEH) C_{18} column ($2.1 \times 50\ \text{mm}$, $1.7\ \mu\text{M}$ particle size) and Waters Quattro Premier XE MS were used to separate and analyze the compounds. The MS was operated in positive ion mode using electrospray ionization (ESI). Waters MassLynx 4.1 and QuanLynx 4.1 software were used for data acquisition and analysis. The mobile phases consisted of 0.1% vol/vol formic acid and 2 mM ammonium acetate in water (solvent A) and 0.1% vol/vol formic acid and 2 mM ammonium acetate in methanol (solvent B). The flow rate was 0.3 ml/min. The mobile-phase gradient started at 90% solvent A and 10% solvent B. The mobile phase was then changed to 90% solvent B in 3 min with the following power-law function (curve 9 in Waters MassLynx software).

$$C(t) = C_i + [(C_f - C_i) * (X^n)] \quad (1)$$

where $X = (t - T_i)/(T_f - T_i)$, $n = 5$ (for curve 9), $C(t)$ is the instantaneous composition at time (t), C_i is the composition of B at the beginning of the segment, C_f is the composition at the end of the segment, and T is time.

Finally, the mobile phase was abruptly switched to 90% A and 10% B for 1.5 min. The column temperature was 50°C , and the autosampler temperature was 10°C . For all samples, the injection volume was $10\ \mu\text{l}$. For the MS/MS, the detector capillary voltage was 3.3 kV, and the extractor voltage was 3 V. The source and desolvation temperatures were 125°C and 400°C , respectively. Nitrogen was used as the

cone and desolvation gas and set at flow rates of 150 liters/h and 800 liters/h, respectively. The retention times (RT) and MS/MS parameters for each antibiotic were determined using the Quanopt function in the Waters MassLynx software (Table 1). The limits of detection (LOD) and limit of quantification (LOQ) of the external standards were calculated as follows.

$$\text{LOD or LOQ} = X\sigma/S \quad (2)$$

where $X = 3$ for the LOD or $X = 10$ for the LOQ, σ = the standard deviation of the response from three independent LC-MS runs, and S = the slope of the calibration curve.

Xevo TQ-XS optimization. Standards were shipped overnight on wet ice to the Waters Demo Laboratory (Beverly, MA) to optimize on the Acquity UPLC Xevo TQ-XS. The Xevo column was the same (Acquity UPLC BEH C_{18} , 1.7 μm ; 2.1 mm by 50 mm), but the mobile phase consisted of 0.3% formic acid in water (solvent A) and 0.3% formic acid in acetonitrile (solvent B). The mobile phase gradient started at 98% solvent A and 2% solvent B. The mobile phase was then switched to 10% solvent A and 90% solvent B in 3 min with curve 6 in Waters MassLynx ($n = 1$ in equation 1). The mobile phase was abruptly switched to 98% A and 2% B for the last 1.5 min. The injection volume was 0.5 μl , and the detector gain was set to 0.1. The positive ion capillary was 0.5 kV, and the cone voltage was 50 V. The desolvation gas and cone gas (nitrogen) flow rates were 1,000 liters/h and 150 liters/h. The desolvation temperature was 600°C, and the source temperature was 150°C. The LC-MS parameters of all of the antibiotics, except for colistin and vancomycin, were determined with IntelliStart optimization with Waters MassLynx software. The MS methods for colistin and vancomycin were determined by manually adjusting the cone voltage and capillary voltage and scanning the product ion spectra. The $M + 2H$ ions were used for MRM of colistin and vancomycin (Table 1).

Comparison of extraction solvents in artificial sputum medium. To compare the extraction efficiency of three solvents, antibiotics were spiked into ASM (Text S1 in the supplemental material) (73) at concentrations of 0, 0.14, 0.41, 0.123, 0.370, 1.1, 3.3, and 10 μM . The three extraction solvents were 1% DTT, methanol, or 16/84 acetonitrile/2% acetic acid. Each solvent was spiked with 1.33 μM of both internal standards, linezolid- d_3 , and levofloxacin- d_6 . Solvent (150 μl) was added to 50 μl of ASM. The samples were vortexed for 30 s, shaken at 4°C on a shaking platform with moderate agitation for 15 min, and centrifuged at 13,200 relative centrifugal force (RCF) for 10 min at 4°C. The supernatant was pipetted into amber glass vials and injected directly into the LC-MS.

The apparent limit of detection (ALOD) was calculated for each antibiotic with each extraction solvent. The ALOD was calculated similarly as the LOD (equation 2), where σ is the standard deviation of the response from the spiked-in antibiotics, and S is the slope of the linear fit for the antibiotic spike-in response versus spiked-in concentration. To visualize the relationship between the recovered antibiotic concentration and the known spiked-in concentration, a linear model was fitted to the recovery data from three independent experiments for each extraction solvent. The coefficient of variation (COV) for three experiments was calculated as the standard deviation of the recovered concentrations divided by the mean of the recovered concentrations. A COV threshold of 30% was used to identify antibiotics that were reproducibly measured (74).

Accuracy of solvent calibration with sputum samples. The accuracy of our surrogate calibration approach (external standards in water with two internal standards) was determined by spiking 1.6 μM antibiotic standards into three sputum samples (spiked sample). An aliquot of nonspiked sputum sample was also processed because antibiotic-free sputum was not available. The background contributions from endogenous antibiotics were subtracted, and accuracy was calculated as the added concentration minus the measured concentration after background subtraction divided by the added concentration.

Sputum collection and extraction. Sputum samples from 11 subjects with CF were selected from a larger airway microbiome study that was approved by the University of Michigan Medical School Institutional Review Board (HUM00037056). Subjects were 6 males and 5 females, age 21 to 56 years (median 37). Sputum samples were collected by subjects at home and stored at 4°C for up to 23 days. All samples were expectorated sputum. Subjects also completed a daily survey reporting symptoms and antibiotic use of both chronic use maintenance antibiotics and episodic treatment antibiotics prescribed to treat pulmonary exacerbations (75). Samples and surveys were regularly shipped in batches on ice packs to the University of Michigan. Sputum samples were mixed with a transfer pipette, aliquoted, and stored at -80°C . Sputum aliquots were shipped from the University of Michigan to the University of California, Irvine on dry ice and subsequently thawed on ice for additional aliquoting. The sputum was homogenized by vigorous vortexing and pipetting, partitioned into 50- μl aliquots, and refrozen at -80°C . Fifty microliter aliquots of sputum were thawed on ice and extracted using the same method described for the artificial sputum medium spike-in experiments and 1% DTT for extraction solvent. Symptom scores were calculated from the daily surveys as previously described (75).

UPLC-MS/MS data filtering. Peaks acquired on the Quattro Premier were automatically picked and filtered using QuanLynx software. Data were imported into R, and the following criteria for filtering were applied: minimum peak area under the curve of 20 and signal to noise ratio of 10. Because several of the antibiotics had high carryover rates (including ciprofloxacin, levofloxacin, and trimethoprim), sample peaks were also filtered out when the area under the concentration-time curve (AUC) was lower than the wash ran before the sample. Scripts can be found at https://github.com/tgallagh/LCMS_Antibiotics. The Xevo TQ-XS peaks were manually picked and filtered using TargetLynx software.

Data analysis. Contingency tables were constructed to compare the LC-MS and survey data. The surveys were treated as the standard, and sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV) were calculated for each antibiotic. Specifically, the sensitivity is the number of samples where the antibiotic was detected and reported divided by total number of reported

samples. Specificity is the number of samples where the antibiotic was undetected and unreported divided by the total number of unreported samples. PPV is the number of samples where the antibiotic was detected and reported divided by the number of times an antibiotic was detected, and NPV is the number of samples where an antibiotic was unreported and undetected divided by the number of undetected instances. To determine if the means of detected antibiotic concentrations were significantly different between the two instruments, paired Wilcoxon rank sum tests were performed on azithromycin, aztreonam, ceftazidime, ciprofloxacin, sulfamethoxazole, tobramycin, and trimethoprim using the “wilcox.test” function in R. Pearson’s correlations between negatives or positives with subject and sample data were completed using the “cor.test” function.

Data availability. The raw Quattro Premier LC-MS data files can be found on Metabolomics Workbench study ST001365. Intermediate data files for the Quattro Premier and Xevo are available at https://github.com/tgallagh/LCMS_Antibiotics.

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

SUPPLEMENTAL FILE 1, XLSX file, 0.01 MB.

SUPPLEMENTAL FILE 2, XLSX file, 0.01 MB.

SUPPLEMENTAL FILE 3, XLSX file, 0.04 MB.

SUPPLEMENTAL FILE 4, XLSX file, 0.04 MB.

SUPPLEMENTAL FILE 5, PDF file, 0.7 MB.

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