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Latent introduction to the Netherlands of multiple antibiotic resistance including NDM-1 after hospitalisation in Egypt, August 2013

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We describe the introduction of various multi-drug resistant bacterial strains, including an NDM-1-producing *Klebsiella pneumoniae*, through a traveller returning from Egypt, where they had been admitted to a private hospital. All family members of the patient were colonised with one or more extended-spectrum beta-lactamase producing strains. These findings emphasise the importance of adherence to isolation precautions for returning patients and suggest the need for inclusion of *Enterobacteriaceae* in admission screening.

We here report of a patient who had been hospitalised in Egypt for appendicitis in July 2013, and was colonised with various multiresistant *Enterobacteriaceae* including strains producing NDM-1, oxacillinase-48 (OXA-48) and extended spectrum beta-lactamase (ESBL). Explorative screening for multiresistant microorganisms among the patient's family members also yielded several ESBL-producing microorganisms. This report addresses the need for heightened awareness of patients and family members who have recently been exposed to healthcare environments in countries with high levels of antibiotic resistance.

Patients repatriated after hospitalisation abroad are a risk for introducing multiresistant microorganisms into hospitals in their home countries. In 2008, New Delhi metallo-beta-lactamase (NDM), which hydrolyses last-line carbapenem antibiotics, has been for the first time described in a Swedish patient returning from India [1]. Most reports on NDM are related to travellers returning from Pakistan and India. However, the global dispersal of NDM is of growing concern [2]. In the past two years, NDM-producing strains have been reported in patients returning from the African continent without obvious links to the Indian subcontinent [3,4].

Case description

A Dutch patient in their 40s was admitted to our hospital for fever and abdominal pain. On admission, computed axial tomography showed a periappendicular abscess. Four days earlier, the patient had returned from holidays in Egypt with his spouse and two children. One week into their holidays (two weeks before presentation at our hospital) the patient had complained about right lower abdominal pain and was admitted to a private hospital in Egypt where 400 mg ciprofloxacin twice a day and 500 mg metronidazole three times a day were given intravenously for two weeks. The patient was discharged after 13 days without having undergone any surgical intervention, and subsequently returned to the Netherlands.

In our hospital, the abscess was drained and the patient was treated with piperacilin/tazobactam 4,500 mg three times a day intravenously for five days with good clinical response.

A perianal screening swab taken on admission grew *Klebsiella pneumoniae*, which was resistant to meropenem (minimum inhibitory concentration: 32 mg/L). Molecular testing of the strain by PCR and sequencing of the PCR product revealed that the strain harboured the gene encoding NDM-1. Molecular testing of faeces detected OXA-48, and culture of this sample grew *Escherichia coli* and *K. pneumoniae* positive for OXA-48.

We also screened the patient's spouse and children, who had been visiting the patient in the hospital in Egypt. Stool samples were obtained 14 days after admission of the index patient. The Table shows an overview of the screening results for multiresistant microorganisms of the family. The patient, his spouse and the youngest child carried ESBL-producing strains with CTX-M1. The older child carried two different ESBL-producing *E. coli* strains positive for CTX-M9. A gene encoding *K. pneumoniae* carbapenemase (KPC) was detected by molecular tests from faeces of the youngest child. The culture of this sample remained negative for carbapenemase-producing strains. Screening of contact patients on the ward where the index patient was treated did not reveal further dissemination of any resistant strains.

Table

Carbapenemase and/or extended-spectrum beta-lactamase-producing strains from samples of a family returning to the Netherlands from Egypt, August 2013

Strain/resistance encoding genes[a]	Index patient	Spouse	Older child	Younger child
Klebsiella pneumoniae/ NDM1, CTX-M1, all TEMs[b]	+			
Escherichia coli/OXA-48, CTX-M1	+			
K. pneumoniae/OXA-48, SHV	+			
E. coli/CTX-M1, all TEMs[b]	+			
E. coli/CTX-M1, all TEMs[b] except for TEM 104				+
E. coli/CTX-M1 (no TEM)	+	+		
E. coli/CTX-M9		+	+, +[c]	
Resistance genes detected directly on stool samples[d]				
OXA-48	+			
KPC				+

[a] Strains phenotypically non-susceptible to third generation cephalosporins and/or carbapenems were tested for resistance genes by microarray Checkpoint chip CT103 (Checkpoints, Wageningen, the Netherlands). Detected ESBL-encoding genes (TEM-104, TEM-164, TEM-238, SHV, CTX-M) and carbapenemase-encoding genes (KPC, NDM, OXA-48, VIM, IMP) are reported.

[b] 'All TEMs' refers to detection of TEM-104, TEM-164 and TEM-238.

[c] Two CTX–M9 producing *E. coli* strains were found, which were phenotypically different and had distinct antimicrobial resistance patterns.

[d] Stool samples of all family members were tested for carbapenemase-encoding genes (KPC, OXA-48, VIM, NDM) by the microarrays Check-Direct CPE (Checkpoints, Wageningen, the Netherlands) and Hyplex Superbug ID (Amplex Biosystems GmbH, Gießen Germany).

Discussion

High prevalence of ESBL-producing bacteria with rates of over 60% in Egyptian hospitals has been reported [5,6]. Also outbreaks involving OXA-48- and VIM-1 carbapenemase-producing strains have been described in the southern Mediterranean region including Egypt [7]. The occurrence of an NDM-1-producing *Acinetobacter* in Egypt has previously been reported, but so far no reports of NDM-1-producing *Enterobacteriaceae* could be found in the literature [8]. This is in contrast to other countries in the area such as Morocco and the United Arab Emirates where these bacteria have already been isolated [9-11].

Highly sensitive and rapid screening methods are the mainstay to prevent introduction of multiresistant microorganisms in hospitals in low prevalence countries by repatriated patients. Molecular tests that allow detection of carbapenemase-encoding genes directly from clinical samples are promising when used in addition to conventional culturing [12].

In the youngest child, we detected a KPC gene by two molecular testing methods directly from faeces, but we were not able to confirm these findings by culture. The bacterial load of the KPC- producing strain was probably too low to be cultured.

It is well recognised that repatriated patients are a risk for introducing multiresistant microorganisms. Family members attending patients hospitalised abroad may also be at risk of acquiring multiresistant bacteria, as our case illustrates. Although we cannot be sure that the family members picked up all strains in the hospital in Egypt, the diversity of multiresistant microorganisms including strains rarely found in the Netherlands makes this highly plausible. Neither can we rule out that the strains were acquired in Egypt outside the hospital, since travelling to African countries has been described as a risk factor for ESBL carriage [13].

Based on these findings, we recommend alertness and compliance with isolation precautions. Infection control guidelines may need to be expanded to include admission screening for resistant *Enterobacteriaceae* in low prevalent counties for a growing number of international travellers, including family members who have visited a patient abroad.

Conflict of interest

None declared.

Authors' contributions

EB collected the data and drafted the manuscript; AWF participated in the coordination and concept of the manuscript; KZ performed and analysed the molecular tests; JPA participated in the coordination and concept of the manuscript and helped with the draft of the manuscript; DB organised the sample collection and participated in the concept of the manuscript; HG coordinated and edited the manuscript; JWR supervised the molecular research and analysis.

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Multidrug-resistant tuberculosis in Uzbekistan: results of a nationwide survey, 2010 to 2011

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Multidrug-resistant tuberculosis (MDR-TB; resistance to at least rifampicin and isoniazid) is a global public health concern. In 2010–2011, Uzbekistan, in central Asia, conducted its first countrywide survey to determine the prevalence of MDR-TB among TB patients. The proportion of MDR-TB among new and previously treated TB patients throughout the country was measured and risk factors for MDR-TB explored. A total of 1,037 patients were included. MDR-TB was detected in 165 treatment-naïve (23.2%; 95% confidence interval (CI) 17.8%–29.5%) and 207 previously treated (62.0%; 95% CI: 52.5%–70.7%) patients. In 5.3% (95% CI: 3.1%–8.4%) of MDR-TB cases, resistance to fluoroquinolones and second-line injectable drugs (extensively drug resistant TB; XDR-TB) was detected. MDR-TB was significantly associated with age under 45 years (adjusted odds ratio: 2.24; 95% CI: 1.45–3.45), imprisonment (1.93; 95% CI: 1.01–3.70), previous treatment (4.45; 95% CI: 2.66–7.43), and not owning a home (1.79; 95% CI: 1.01–3.16). MDR-TB estimates for Uzbekistan are among the highest reported in former Soviet Union countries. Efforts to diagnose, treat and prevent spread of MDR-TB need scaling up.

Introduction

Tuberculosis (TB) remains a major challenge to public health worldwide. In 2011, the World Health Organization (WHO) estimated that there were about 12 million prevalent TB cases globally of whom about 630,000 were infected with strains resistant to at least both rifampicin and isoniazid (multidrug-resistant TB; MDR-TB) [1]. Inadequate use of anti-TB drugs to treat drug-susceptible TB favours the emergence of drug resistance. MDR-TB patients require combination treatment which commonly lasts 20 months or more, employing drugs which are difficult to procure and more toxic and expensive than those used to treat drug-susceptible forms of the disease [2-4].

Over the last decade, countries of the former Soviet Union have reported the highest levels of MDR-TB infection among TB patients [5,6]. In one of these countries, Uzbekistan, in central Asia, two sub-national studies performed since 2001 have detected MDR-TB in 13–15% of new TB patients and in 40–60% of previously treated patients [7,8]. In 2011 Uzbekistan had a population of 29 million inhabitants and a Gross National Income of EUR 1,137 per capita, making it a lower middle income country [9]. The state Republican DOTS Centre (RDC) in the capital city Tashkent is responsible for the TB control services in all of the country's 14 administrative divisions, including the Autonomous Republic of Karakalpakstan. In 2011, Uzbekistan reported 15,913 TB cases, 28% of whom were culture-confirmed and 50% of these also had results for drug-susceptibility testing (DST) to both isoniazid and rifampicin [10].

As estimates from subnational surveys may not necessarily be generalisable to a whole country, the RDC conducted the first nationally-representative study of anti-TB drug resistance based on a countrywide sample of TB patients in Uzbekistan. The estimate from this study would enable the national TB control programme and its partners, particularly Médecins Sans Frontières (MSF) and the Global Fund against AIDS, TB and Malaria (TGF), to better target their efforts and resources and understand the different risk factors associated with MDR-TB, including co-infection with human immunodeficiency virus (HIV). In this article we describe the main outcomes of the survey and we

discuss their implications for the continued surveillance, prevention, diagnosis, treatment and scale-up of MDR-TB programme management in the country. We believe the findings of this study are relevant beyond the country's borders as they add to the current knowledge base about the burden of MDR-TB in eastern Europe [11], a phenomenon that has a sizeable impact upon the epidemiology of TB in European Union countries as a result of migration and population movements [12].

Methods

Sampling, recruitment and statistical analysis

The objectives of the study were to estimate (i) the proportion of new (TB treatment-naïve) and previously treated (four weeks of treatment or more) TB cases infected with MDR-TB strains; (ii) the proportion of MDR-TB cases infected with strains additionally resistant to fluoroquinolones and second-line injectable drugs (extensively drug-resistant TB; XDR-TB [13]); and (iii) the magnitude of association between risk factors and infection with MDR-TB strains. Definitions used for previous treatment history and resistance patterns were as recommended by WHO [14]. A detailed survey protocol including associated costs was prepared according to these WHO guidelines and approved by the National Ethics Committee of the Ministry of Health.

The survey was carried out using non-invasive techniques employed in the course of regular investigation of any patient with presumptive TB. Patients were asked to sign their informed consent for participation. Confidentiality was respected during collection and storage of clinical data.

The sampling frame consisted of pulmonary TB patients diagnosed at public healthcare facilities in all administrative regions of Uzbekistan. All patients diagnosed with positive sputum smear on microscopy were eligible for inclusion. The sample size was calculated on the basis of new sputum-smear TB cases notified in the country in 2008 (n=5,234), an expected prevalence of 15% MDR-TB among new cases (based on the most recent survey in Tashkent) [8], an absolute precision of 2.5% of the expected prevalence, and a 95% confidence level. A total of 818 new cases were thus required, inclusive of a 20% margin to cover for expected losses. As all previously treated sputum-smear positive TB cases presenting to healthcare facilities during the survey period were also to be recruited, 1,174 study subjects were targeted in total.

The survey was carried out over the 12 months until June 2011 using 100% sampling. The national sample was split among the different regions (Oblasts) according to the TB notification patterns from 2008. During the study period, eligible patients were referred from the districts to the regional-level TB dispensaries for enrolment, collection of sputa for the study and interviewing until the expected number of cases from each region was reached. Cases were excluded if they were sputum smear-positive at the end of a re-treatment regimen, or if sputum was collected 10 days or more into the current course of treatment, or if they did not or could not consent, or if they were not smear-positive, or were later found to be infected with strains other than *Mycobacterium tuberculosis* complex. All patients were offered HIV testing and counselling. A standardised information form was used to collect data (Figure 1). Information was cross-checked with patient records when available and then transferred from the completed forms onto two electronic registers housed at the National TB Reference Laboratory (NRL) and the RDC and then merged.

The proportions used to describe the first-line drug resistance patterns among new and previously treated cases tested were weighted by the TB cases notified in each administrative region in 2011. Univariate analysis was used to study relationships between risk factors and MDR-TB, and associations with $p<0.10$ were further analysed using multivariable logistic regression. Effects were expressed as crude and adjusted odds ratios and $p<0.05$ was considered statistically significant. The associations were further explored by imputing missing values and by using random-effects modelling. Statistical analysis was performed using STATA SE/12.1 (StataCorp LP, TX, USA) and the ggplot2 package running in the R environment (R 3.0.1; [15]) was used to produce all graphics.

Laboratory procedures

The country is covered by a network of over 320 laboratories which perform direct microscopy of sputum at primary healthcare level. Two laboratories in the country can perform both culture and DST for first- and second-line anti-TB drugs reliably: the NRL in Tashkent and the laboratory at the TB hospital in Nukus, Karakalpakstan. Both laboratories perform DST on solid and liquid media, line-probe assay (LPA) and participate in quality assurance programmes with the WHO supranational TB reference laboratories (SRL) in Borstel and Gauting, Germany. Laboratory capacity to detect drug-resistance has increased substantially in Uzbekistan since 2008 and more than a thousand MDR-TB cases are now detected each year in the country [1].

For the purposes of the survey, 3 to 5 ml of sputum were collected from each study participant. If inspection of the specimen showed saliva or insufficient quantity, the patient was asked to provide a morning sputum the following day. Patients whose sputum was found to be smear–positive at the district or regional level laboratories using bright-field microscopy after hot Ziehl-Neelsen staining, were requested to provide a second sputum specimen. The second specimens were stored between 4°C and 12°C for a maximum of six days before shipment to the NRL in Tashkent where they were rechecked using fluorescence microscopy after Auramine O staining. Specimens from Karakalpakstan

Figure 1

Clinical Information Form[a], nationwide study on drug-resistant tuberculosis, Uzbekistan, 2010–2011

Part 1. Details of the TB patient	1.	DRS code	
	2.	Region	
	3.	District	
	4.	Patient surname	
	5.	Patient name	
	6.	Date of birth	day/month/year
	7.	Sex	male/female
	8.	Date of registration in the TB register	day/month/year
	9.	HIV status	
Part 2. Clinical information	10.	Have you ever been treated for TB previously?	No (if not, ask questions 11-16) Yes (if yes, go to question 17)
	11.	How long have you been sick?	
	12.	Did you have the same symptoms prior to this episode?	
	13.	Did you have other symptoms of lung disease prior to this episode (haemoptysis, chest pain, cough)?	
	14.	Did you have sputum examination prior to this episode?	
	15.	Did you ever take anti-TB drugs for more than one month?	
	16.	Did you ever have injections for more than one month?	
	17.	Did the patient remember previous treatment for TB after these questions?	
Part 3. Medical records and the final decision on previous anti-TB treatment	18.	After checking through the available medical files have you discovered that the patient was registered for TB treatment before?	No Yes (If yes, number in the TB03 register)
	19.	Final decision taking into consideration the answers of the patient on the standardised clinical history: Has the patient been previously treated for TB for more than one month?	No (go to question 21) Yes (go to question 20) Unknown
	20.	If yes, what was the outcome of previous treatment?	Cured/treatment completed Failed new patient regimen using first-line drugs only Failed retreatment regimen using first line drugs only Failed regimen including second-line drugs Defaulted Other
Part 4. Social determinants	21.	Country of birth?	Uzbekistan Abroad (specify the country)
	22.	Your education (completed)?	Higher (University) Middle special (Technical college) Secondary Primary or lower
	23.	Your housing conditions?	House (apartment) owned House (apartment) rented Dormitory Homeless
	24.	How many people share your house?	Specify the number of persons
	25.	Your occupation?	student unemployed / looking for a job invalid retired occupation (specify)
	26.	Have you been in prison in the last 10 years?	No Yes (specify the number of years)
	27.	Have you travelled abroad for work in the last 2 years?	No Yes (specify the country)
	28.	How often do you take alcohol?	never rarely sometimes often
	29.	Have you been smoking daily during the past 5 years?	No Yes
	30.	Have you taken any illicit drugs during the last month?	No Yes If yes, which drug:
	31.	Have you been in hospital in the last 10 years?	No Yes If yes, i. for how many weeks ii. which hospital

Name of the responsible officer

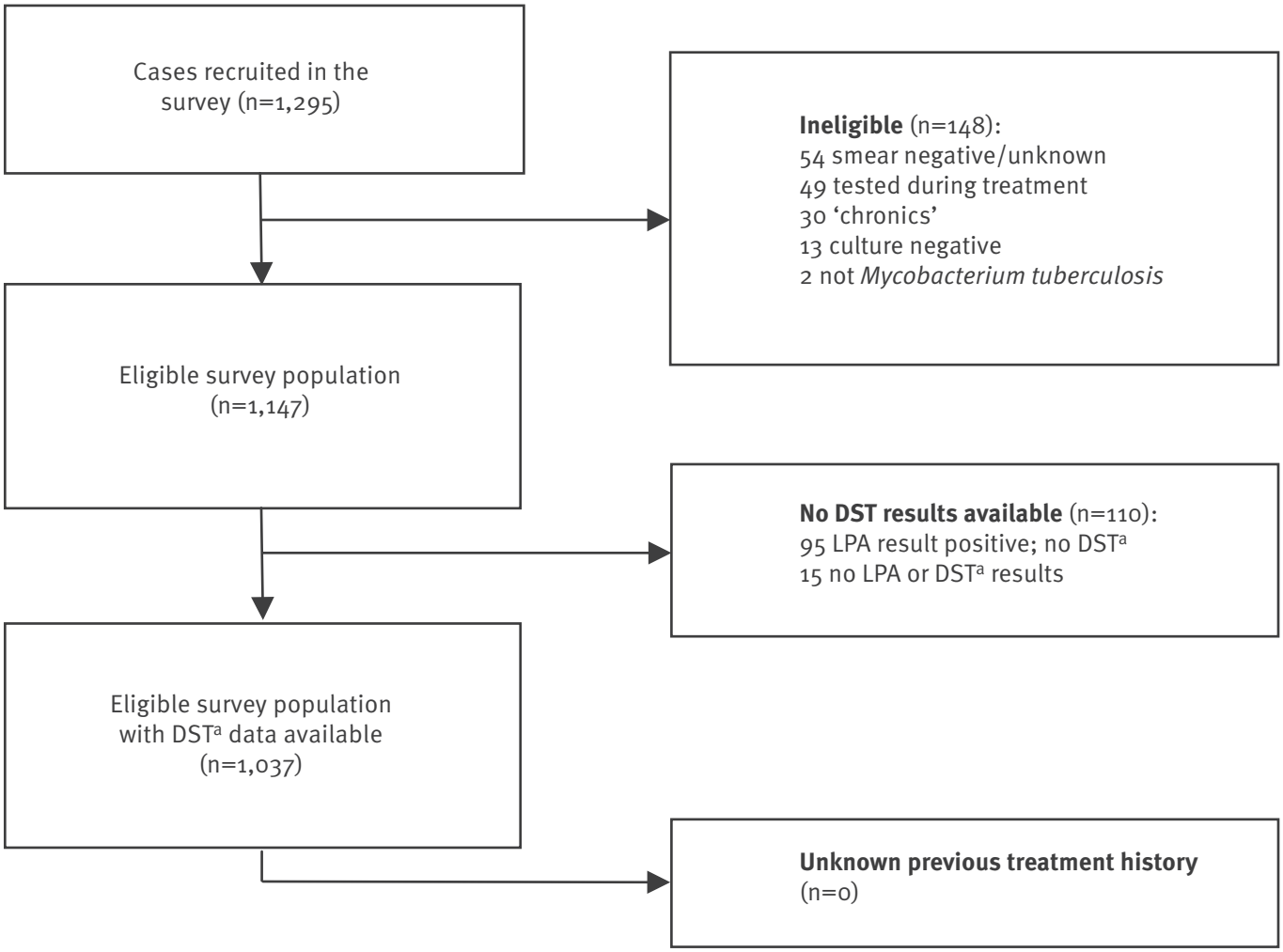
_____

Date of completion ___/___/_____

[a] Translated from Russian.

Figure 2

Flowchart of patients included in the nationwide study on drug-resistant tuberculosis, Uzbekistan, 2010–2011



DST: drug-susceptibility testing; LPA: line-probe assay.

[a] For isoniazid and rifampicin (phenotypic).

and Khorezm were processed identically at the Nukus laboratory. Patients were considered smear-positive if at least one of two sputum specimens examined yielded ≥ 1 acid fast bacillus per 100 high power fields [16]. All specimens were processed using a modified Petroff-method or NALC-NaOH procedures and inoculated in both Löwenstein-Jensen medium and mycobacteria growth indicator tube (MGIT) liquid medium (Becton-Dickinson, New Jersey, United States). DST was performed using the method of proportion [17] and the MGIT system. Susceptibility patterns to isoniazid, rifampicin, ethambutol and streptomycin were determined for all cases. If the growth of isolates allowed, MDR-TB strains were additionally tested for ofloxacin, a second-line aminoglycoside (amikacin and/or kanamycin) and capreomycin. Positive cultures were tested using Genotype MTBDR*plus* (1st generation, Hain Lifescience, Nehren, Germany) for identification of *M. tuberculosis* complex and detection of resistance-conferring mutations to isoniazid and rifampicin. Each isolate, for which the results of genetic and phenotypic DST assays differed, was transferred to the SRL for determination of the final susceptibility pattern. If genetic mutations known to be associated with drug resistance were identified using Genotype MTBDR*plus*, the isolate was considered resistant to the respective drug. If no mutation was identified, the SRL repeated the DST in liquid medium and this result was considered definitive.

Quality control of DST was provided through (i) internal control according to EN DIN 58943-8:2009-04, (ii) four monitoring visits by an expert from the SRL München-Gauting (co-author H.H.), (iii) annual external quality assessment by testing a panel of 30 WHO control strains (provided by the Institute of Tropical

Table 1A

Characteristics of the study population, completeness of drug-susceptibility testing data and associations with multidrug-resistant tuberculosis in the nationwide study on drug-resistant tuberculosis, Uzbekistan, 2010–2011 (n=1,037)

Characteristic	New		Previously treated		Total		Missing data for DST[a] to isoniazid and rifampicin		Association with missingness, Chi-square, P-value	MDR-TB, %	Odds ratios for MDR-TB, univariate analysis (95%CIs)	Adjusted odds ratios for MDR-TB, multivariable regression (95%CIs)
	n	%	n	%	n	%[b]	n	%				
Sex												
Female	364	72	141	28	505	44	53	10	0.85, P=0.36	35	Reference	NA
Male	418	65	224	35	642	56	57	9		36	1.06 (0.82-1.36)	NA
Age												
>44 years	332	72	130	28	462	40	43	9	0.07, P=0.79	26	Reference	Reference
<45 years	450	66	235	34	685	60	67	10		43	2.11 (1.61-2.76)	2.24 (1.45-3.45)
Administrative region/city												
Andijan	81	82	18	18	99	9	2	2	185.9, P=0.00	30	Reference	Reference
Bukhara	22	55	18	45	40	3	0	0		40	1.56 (0.73-3.37)	1.27 (1.10-1.46)
Djizak	26	62	16	38	42	4	1	2		37	1.35 (0.63-2.92)	1.05 (0.80-1.39)
Fergana	109	87	16	13	125	11	16	13		28	0.89 (0.49-1.63)	1.07 (0.91-1.27)
Karakalpakstan	61	46	71	54	132	12	19	14		62	3.82 (2.14-6.80)	2.33 (1.85-2.94)
Kashkadarya	59	70	25	30	84	7	35	42		51	2.44 (1.20-4.96)	2.17 (1.60-2.95)
Khorezm	31	65	17	35	48	4	6	13		33	1.17 (0.54-2.54)	0.73 (0.60-0.89)
Namangan	63	73	23	27	86	7	9	10		32	1.13 (0.59-2.15)	1.08 (0.87-1.33)
Navoiy	17	44	22	56	39	3	1	3		42	1.71 (0.78-3.71)	0.71 (0.52-0.97)
Samarkand	96	77	28	23	124	11	1	1		20	0.57 (0.30-1.06)	0.46 (0.35-0.61)
Surhadarya	43	81	10	19	53	5	17	32		17	0.47 (0.18-1.25)	0.44 (0.34-0.56)
Syrdarya	32	68	15	32	47	4	0	0		36	1.33 (0.64-2.78)	1.68 (1.39-2.04)
Tashkent city	67	54	57	46	124	11	3	2		38	1.44 (0.81-2.54)	0.93 (0.79-1.09)
Tashkent region	75	72	29	28	104	9	0	0		38	1.41 (0.78-2.53)	1.19 (0.95-1.50)
Origin												
Uzbek	764	68	360	32	1124	98	108	10	0.02, P=0.88	36	Reference	NA
Foreign	18	78	5	22	23	2	2	9		29	0.71 (0.27-1.85)	NA
Education												
Higher	242	66	125	34	367	32	24	7	5.79, P=0.02	38	Reference	NA
Up to secondary	540	69	240	31	780	68	86	11		35	0.85 (0.65-1.10)	NA
Home-owner												
No	102	63	59	37	161	14	22	14	3.58, P=0.06	43	Reference	Reference
Yes	680	69	306	31	986	86	88	9		35	0.70 (0.49-1.01)	0.56 (0.32-0.99)
Occupation												
Not employed	565	66	295	34	860	75	86	10	0.67, P=0.41	38	Reference	Reference
Employed/ Student	217	76	70	24	287	25	24	8		30	0.72 (0.53-0.97)	0.67 (0.37-1.22)
In prison in the previous 10 years												
No	759	70	319	30	1078	94	106	10	1.22, P=0.27	34	Reference	Reference
Yes	23	33	46	67	69	6	4	6		60	2.88 (1.72-4.81)	1.93 (1.01-3.70)
Hospitalised in the last 10 years												
No	594	91	61	9	655	57	58	9	0.95, P=0.33	26	Reference	Reference
Yes	188	38	304	62	492	43	52	11		49	2.77 (2.14-3.60)	1.29 (0.78-2.15)

CI: confidence interval; DST: drug-susceptibility testing; MDR-TB: multidrug-resistant tuberculosis; NA: not applicable.

[a] For isoniazid and rifampicin.

[b] For the column.

Table 1B

Characteristics of the study population, completeness of drug-susceptibility testing data and associations with multidrug-resistant tuberculosis in the nationwide study on drug-resistant tuberculosis, Uzbekistan, 2010–2011 (n=1,037)

Characteristic	New		Previously treated		Total		Missing data for DST[a] to isoniazid and rifampicin		Association with missingness, Chi-square, P-value	MDR-TB, %	Odds ratios for MDR-TB, univariate analysis (95%CIs)	Adjusted odds ratios for MDR-TB, multivariable regression (95%CIs)
	n	%	n	%	n	%[b]	n	%				
Worked abroad in the last 2 years												
No	705	68	328	32	1033	90	95	9	1.86, P=0.17	36	Reference	NA
Yes	77	68	37	32	114	10	15	13		37	1.07 (0.70-1.65)	NA
Uses alcohol												
Never	480	70	206	30	686	60	67	10	0.06, P=0.80	36	Reference	NA
Yes	302	66	159	34	461	40	43	9		36	1.00 (0.77-1.30)	NA
Regular smoker												
No	640	71	268	29	908	79	99	11	8.65, P=0.00	35	Reference	NA
Yes	142	59	97	41	239	21	11	5		38	1.13 (0.84-1.54)	NA
HIV infection												
No	707	68	334	32	1041	91	106	10	4.85, P=0.09	32	Reference	NA
Yes	18	55	15	45	33	3	2	6		42	1.45 (0.71-2.99)	NA
Unknown	57	78	16	22	73	6	2	3		27	0.69 (0.41-1.18)	NA
Previously treated for TB												
No					782	68	77	10	0.19, P=0.66	23	Reference	Reference
Yes					365	32	33	9		62	5.42 (4.09-7.19)	4.45 (2.66-7.43)

CI: confidence interval; DST: drug-susceptibility testing; HIV: human immunodeficiency virus; MDR-TB: multidrug-resistant tuberculosis; NA: not applicable.

[a] For isoniazid and rifampicin.

[b] For the column.

Medicine in Antwerp, Belgium), and (iv) re-checking by the SRL of a randomly-selected sample of all isolates collected for the survey. A total of 90 MDR-TB isolates, 104 fully susceptible ones, 102 isoniazid-resistant and rifampicin-susceptible ones, and two isoniazid-susceptible and rifampicin-resistant were rechecked using WHO-recommended sampling parameters (14). This control revealed 7/298 (2.3%) errors for isoniazid, 3/298 (1.0%) for rifampicin, 0/90 (0%) for ofloxacin, 6/90 (6.7%), 4/90 (4.4%) for kanamycin, and 5/90 (5.6%) for capreomycin.

Results

A total of 1,147 eligible culture-positive cases remained in the study, after exclusion of ineligible cases included in the original dataset (Figure 2). The distribution of cases recruited in each of the administrative divisions in the country ranged from 3 to 12% of the total (Table 1). The ratio of new cases enrolled in the survey to those actually notified in each administrative region also varied, from 11.6% in Karakalpakstan to 28.3% in Syrdarya (Figure 3). The majority of cases were male (642; 56%), under 45 years of age (685; 60%), native Uzbeks (1,124; 98%) and had not been previously treated (782; 68%). Thirty-two percent of cases (367) had attended university or a technical college, 86% (986) lived in a home they owned, 25% (287) were employed or studying, 6% (69) had a history of imprisonment and 43% (492) had been hospitalised in the previous 10 years. Intake of alcohol in the previous month was reported to be 'sometimes' or 'often' by 40% of patients (461), and 21% (239) had smoked regularly in the previous five years. HIV-infection was detected in 3% of cases (33).

Of the 1,147 cases, 1,037 (90%) had DST results for all the four first-line drugs tested (isoniazid, rifampicin, ethambutol and streptomycin). Most cases with missing DST had a positive LPA test (95/110; 86%), while in the rest no DST result could be traced. The availability of DST results did not differ significantly by the patient characteristics studied (Table 1).

Resistance patterns by treatment history

Table 2 shows the distribution of resistance to the four first-line drugs among new and previously treated

patients for all 1,037 patients with DST results (percentages weighted for variations in notifications). Any resistance to one or more drugs was observed in 47% of new cases and 82% of previously treated, most often for isoniazid (42% in new and 79% in previously treated) or streptomycin (40% and 77% respectively). Most cases with rifampicin resistance were also resistant to the other three drugs (251/382) or to isoniazid and streptomycin (112), with only few cases (7) being mono-resistant to rifampicin. MDR-TB was detected in 372 cases (23% of new and 62% of previously treated cases). The percentage MDR-TB in both new and previously treated cases did not vary significantly when the analysis was repeated after imputing data for the 110 cases with missing DST results. A total of 14 MDR-TB cases (3.8%) were HIV-infected.

There were 319 MDR-TB cases (86%) with DST results for at least one fluoroquinolone and one second-line injectable drug (Table 3). Of these, 17 (5.3%; 95% CI 3.1%-8.4%) were resistant to both and thus XDR-TB. In addition, 23 cases (7.2%) were resistant only to fluoroquinolones and another 63 (19.7%) to second-line injectable drugs only. The prevalence of second-line drug resistance was comparable between new and previously treated cases. In 15 of 17 XDR-TB cases resistance was observed to all three second-line injectable drugs tested. None of the XDR-TB cases were HIV-infected.

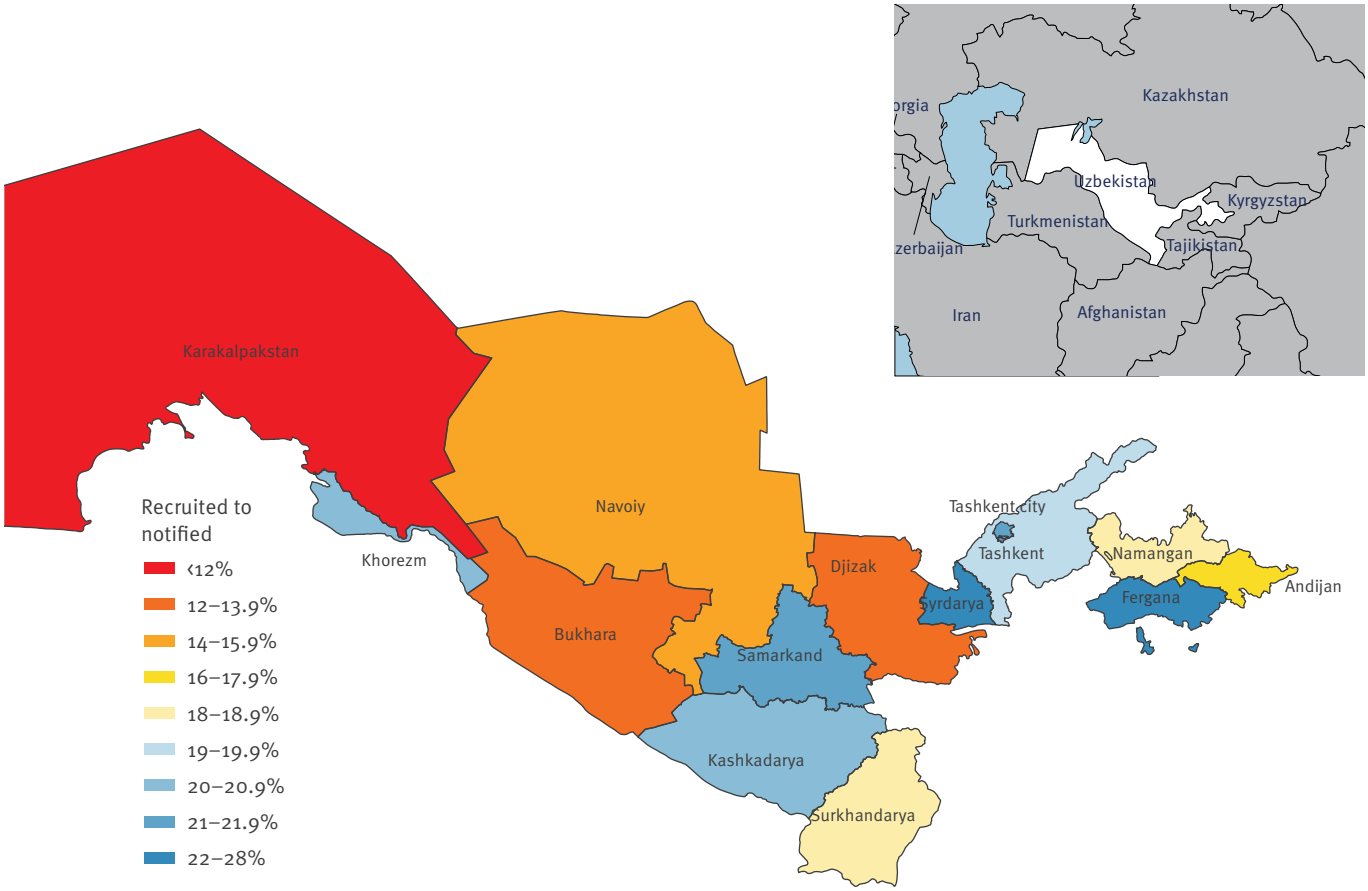
Risk factors associated with infection with MDR-TB strains

At univariate analysis, significant positive associations with MDR-TB were observed with cases reported from Karakalpakstan and Kashkadarya (referenced to Andijan), age under 45 years, history of imprisonment or hospitalisation in the previous 10 years, and previous exposure to anti-TB treatment (Table 1). The percentage of MDR-TB was significantly higher in Karakalpakstan compared to Samarkand and Surhadarya, for new cases, and to Namangan for those previously treated (Figure 4).

When multivariable logistic regression was performed, MDR-TB was positively associated with age under 45

Figure 3

Ratio of new sputum-smear positive tuberculosis cases recruited in the survey (n=782) to those notified in 2011, by administrative region, in the nationwide study on drug-resistant tuberculosis, Uzbekistan, 2010–2011



Countrywide value: 18.6%.

Table 2

Drug-susceptibility results to first-line anti-tuberculosis drugs in the nationwide study on drug-resistant tuberculosis, Uzbekistan, 2010–2011 (n=1,037)

Resistance pattern	Treatment history[a]			
	New cases (n=705)		Previously treated cases (n=332)	
	%[b]	95% CIs[b]	%[b]	95% CIs[b]
Any resistance to isoniazid (H)	41.9	(36.2-47.8)	78.9	(70.4- 85.5)
Any resistance to rifampicin (R)	24.2	(18.6-30.8)	62.5	(53.0-71.1)
Any resistance to ethambutol (E)	20.1	(15.6-25.5)	47.6	(39.9-55.4)
Any resistance to streptomycin (S)	40.0	(33.1-47.4)	76.8	(65.9-85.0)
Total any resistance	**47.4**	**(41.9-52.9)**	**82.0**	**(71.6-89.1)**
Resistance to H only	5.5	(3.3- 8.9)	3.4	(2.0-5.7)
Resistance to R only	0.9	(0.4-1.7)	0.2	(0.0-1.6)
Resistance to E only	0.0	NA	0.2	(0.0-2.0)
Resistance to S only	4.4	(2.5- 7.5)	2.0	(0.9-4.7)
Total mono-resistance	**10.0**	**(7.3-15.5)**	**5.8**	**(3.9-8.5)**
H + R	0.2	(0.0-1.4)	0.9	(0.2-3.4)
H + R + E	0.3	(0.1-1.1)	0.3	(0.1-2.3)
H + R + S	6.9	(4.7-10.2)	17.2	(11.4-25.1)
H + R + E + S	15.8	(12.1-20.4)	43.6	(36.4-51.2)
Total multidrug resistance (MDR)	**23.2**	**(17.8-29.5)**	**62.0**	**(52.5-70.7)**
H + E	0.6	(0.1-2.4)	0.2	(0.0-1.9)
H + S	9.3	(7.1-12.1)	10.6	(8.5-13.2)
H + E + S	3.4	(2.1-5.4)	2.7	(1.1-6.4)
R + E	0.0	NA	0.0	NA
R + S	0.2	(0.0-1.6)	0.2	(0.0-1.5)
R + E + S	0.0	NA	0.2	(0.0-2.0)
E + S	0.1	(0.0-1.1)	0.3	(0.1-2.1)
Total poly-resistance other than MDR	**13.5**	**(10.6-17.1)**	**14.2**	**(11.6-17.1)**
Total susceptible	**52.6**	**(47.1-58.1)**	**18.0**	**(10.9-28.4)**

CI: confidence linterval; DRS: drug-resistance survey; E: ethambutol; H: isoniazid; MDR: multidrug resistance; NA: not applicable; R: rifampicin; S: streptomycin.

[a] Previous treatment history was known for all cases.

[b] Percentages have been weighted by the number of sputum-smear positive TB cases notified in each region in 2011.

years, not living in an owned home, history of imprisonment and previous anti-TB treatment. Regional variations in risk were also observed (Table 1).

Discussion

This is the first nationwide, representative anti-TB drug resistance survey of patients presenting for treatment in Uzbekistan. The proportion of new cases with MDR-TB calculated from this survey is higher than that found in other studies performed in parts of Uzbekistan since 2001. The estimate for MDR-TB in previously treated cases is similar to the one observed in the capital city Tashkent in 2005 [8] but higher than that in Karakalpakstan in 2001-2002 [7].

The proportion of MDR-TB among TB patients in Uzbekistan is among the highest being reported by former Soviet Union countries in recent years [11]. More than two-thirds of MDR-TB cases were resistant to ethambutol and therefore it is likely that in a majority of cases this drug will not be effective if it is added to a second-line drug regimen. HIV infection, which augurs badly for the prognosis in MDR-TB unless managed adequately, was infrequent. It is noteworthy that 44%

Table 3

Drug-susceptibility results to second-line anti-tuberculosis drugs, in the nationwide study on drug-resistant tuberculosis, Uzbekistan, 2010–2011 (n=319)

Resistance pattern	Treatment history[a]					
	New MDR-TB cases			Previously treated MDR-TB cases		
	n	%	95% CIs	n	%	95% CIs
MDR-TB cases with DST results for any fluoroquinolone and any second-line injectable drug[b]	144	NA	NA	175	NA	NA
MDR-TB cases susceptible to both fluoroquinolones and second-line injectable drugs	99	68.8	60.5-76.2	117	66.9	59.4-73.8
MDR-TB cases with any resistance to fluoroquinolones	11	7.6	3.9-13.3	12	6.9	3.6-11.7
MDR-TB cases with any resistance to a second-line injectable drug	26	18.1	12.1-25.3	37	21.1	15.3-27.9
MDR-TB cases resistant to both a fluoroquinolone and a second-line injectable drug (XDR-TB)	8	5.6	2.4-10.7	9	5.1	2.4-9.5

CI: confidence interval; MDR-TB: multidrug-resistant tuberculosis; NA: not applicable; XDR-TB: extensively drug-resistant tuberculosis.

[a] Previous treatment history was known in all cases

[b] Cases tested for fluoroquinolones (one or more from ciprofloxacin, ofloxacin or moxifloxacin) and second-line injectable drugs (amikacin and/or kanamycin plus capreomycin; three cases had a test result for either amikacin/kanamycin or capreomycin only).

of MDR-TB cases detected in this survey were not previously exposed to anti-TB drugs and such cases were reported from all administrative regions. Two-fifths of new MDR-TB cases were in individuals aged between 13 and 30 years, with a male to female ratio of 1.2:1. Drug-resistance in cases not previously exposed to anti-TB drugs generally indicates primary infection with resistant strains and points to shortfalls in prevention. Its widespread occurrence in our study across regions, ages and sexes implies that transmission probably occurs in different settings, such as hospitals, prisons, and households.

Representative surveillance data from a number of settings in the world indicate that on average 9.0% of MDR-TB cases (95% CI: 6.7–11.2) have XDR-TB strains [1]. Among MDR-TB patients in Uzbekistan, the proportion of XDR-TB was lower (5.3%; 95% CI: 3.1%–8.4%) although the confidence interval overlaps with those of the global estimates. Nonetheless, two additional observations about the XDR-TB cases in this study have important inferences. Firstly, unlike the case with MDR-TB, the frequency of infection with XDR-TB strains was not different between new and previously treated MDR-TB patients. This indicates direct transmission of XDR-TB. Secondly, next to all XDR-TB patients were resistant to both classes of second-line injectable drugs, the aminoglycosides and the polypeptide capreomycin. This was higher than was found in a recent review [18]: it means that treatment options for XDR-TB patients are substantially compromised given that the injectable drugs – considered a mainstay of XDR-TB regimens [19] - are likely to be ineffective in most XDR-TB patients in the country.

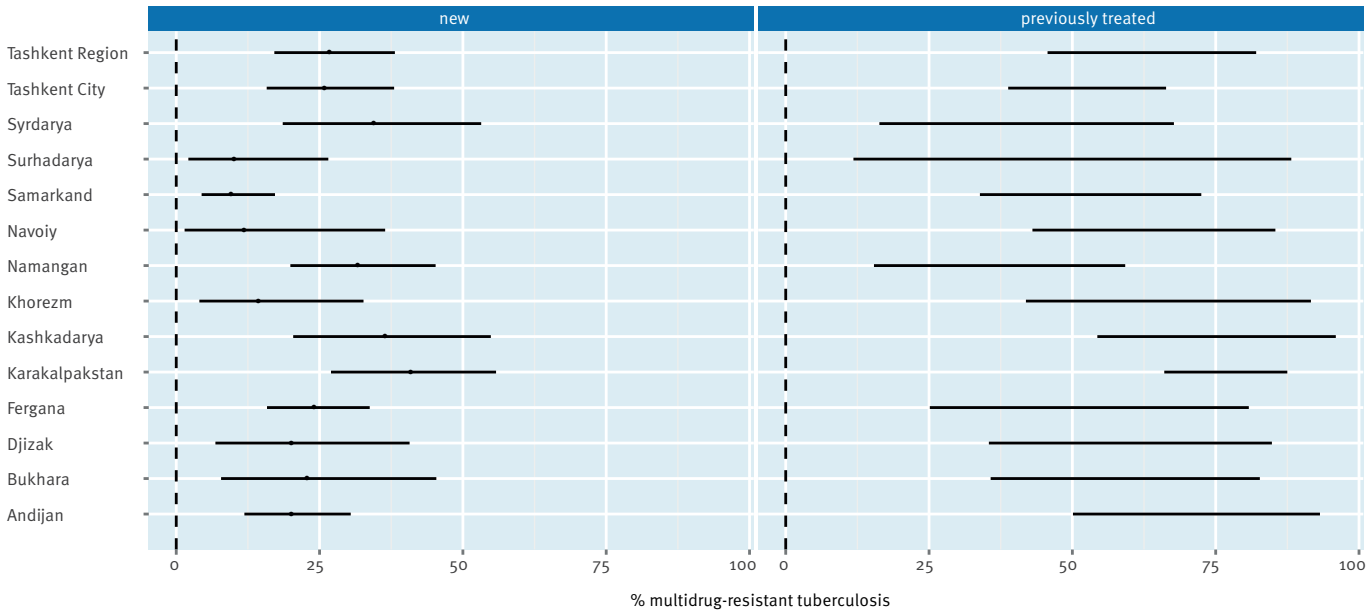
Two social factors remained significantly associated with MDR-TB after adjustment: imprisonment and lack of home-ownership. Incarceration has been associated with MDR-TB elsewhere in the former Soviet Union [20]. This study has now documented this risk in a central Asian setting as well. Individuals who do not possess their house may be more prone to develop MDR-TB as a result of increased mobility and poor treatment adherence. In a subset analysis (data not shown), crude odds for MDR-TB were 1.5 times higher in unemployed patients when compared to those employed. Given that long-standing illness may reduce the chances of employment, this association may be in part an effect of MDR-TB rather than its cause.

Notifications of new sputum-positive TB cases in Uzbekistan have declined each year since 2006, from 7,211 that year to 4,198 in 2012 [1]. There is concern however, that this apparently promising trend may be accompanied by an increase in the prevalence of drug-resistant cases among the residual TB patients. In Uzbekistan, both drug-susceptible and drug-resistant TB patients have been treated in accordance with WHO-recommended guidelines for a number of years. DOTS first-line treatment regimens were piloted in the late 1990s and expanded to reach nationwide coverage by 2005. Since then, treatment success for new sputum-smear positive cases has been reported consistently at around 80%, but is lower in previously treated cases [1].

All patients with MDR-TB detected in this survey were referred for treatment. In some sites, treatment could only be started when second-line drugs became available. Second-line drug treatment for MDR-TB patients is known to comply with international recommendations in

Figure 4

Percentage of multidrug-resistance among new (left) and previously treated (right) tuberculosis cases by region/city in the nationwide study on drug-resistant tuberculosis, Uzbekistan, 2010–2011 (n=1,037)



two treatment sites in Uzbekistan. In 2003, MSF started to support the treatment of drug-resistant TB patients in public healthcare facilities in a project approved by the Green Light Committee in Karakalpakstan [21]. Over 2,300 patients had started treatment by the end of April 2012 of whom over 900 were supported by funding from TGF. Among the 710 MDR-TB patients enrolled between 2003 and 2008, 62% finished their treatment successfully [22]. In Tashkent, the RDC staff have been treating drug-resistant TB patients since 2006 through the support of TGF and UNITAID. By mid-2012, 1,490 MDR-TB patients had started treatment and treatment success for the 446 patients enrolled in 2009 was 61%. Treatment with second-line drugs is also available for imprisoned patients. In 2011, a total of 1,385 MDR-TB cases were detected in Uzbekistan, and 855 were enrolled on treatment. If the estimates observed in this survey are applied to the number of new and previously treated pulmonary TB cases notified in the country in 2011, about 3,000 MDR-TB cases (range: 2,700-3,400) would be expected each year if DST were to be performed on all notified TB cases [1]. About 80% of these cases would be expected to occur among new TB patients. These numbers do not include prevalent MDR-TB cases surviving from previous years and who would likewise be eligible for treatment.

This study had some limitations. While the overall number of cases enrolled in the survey approached the target aimed for at the design stage, the number of cases recruited into the survey as a proportion of cases notified varied between the administrative regions. This may have under- or over-represented the contribution of cases from certain centres to the overall estimate. For this reason, the study estimates were weighted by the TB cases actually notified during the second year of the study (differences between weighted and unweighted values were small). The study was designed to estimate the proportion of MDR-TB among a nationwide sample of new TB cases and was not powered to detect differences between other patient subgroups or the administrative divisions in Uzbekistan. Thus, statistically significant differences could only be discerned between regions with extreme values. The proportion of MDR-TB among previously treated TB cases as estimated in this survey is expected to be conservative given that previously treated patients who experienced a treatment failure in a year prior to the one of the survey were not included.

The validity of data collected by patient interview alone on problem use of alcohol and narcotics was open to question and so these data were not used in the final analysis. This precluded the investigation of associations with these potential risks. The missing data on HIV status in 6% of patients may also have been the reason why no significant relationship was found between HIV infection and MDR-TB, an association which has been noted elsewhere in countries of the former Soviet Union [8]. Although data on DST were missing for 110 eligible cases, when the estimates were recalculated after imputation of missing values only slight differences were noted. The 'missingness' of DST data was therefore considered to be at random. Finally, as the study was limited to sputum-smear positive TB cases, it is possible that the patterns of resistance in children and in sputum-smear negative disease may differ from the ones described in this study.

This survey has identified some useful leads for future study. Geographic disparities in MDR-TB levels may reflect confounding by social variables but also differences in programme performance and variations in the pattern of circulating strains. The lack of association with some of the risk factors measured may be in part due to problems with validity of data and also the relatively small number of observations. These require further study, particularly migration which has been linked to TB in recent reports in central Asia [23,24]. Migration from countries of the former Soviet Union to several European Union countries in the last decade has been marked [25], and studies such as the current one underline the importance of continued surveillance for drug-resistance in the western Europe [12,26]. Biochemical tests and expert interviewers may be needed to elicit valid answers to questions of a sensitive nature such as problem use of alcohol and narcotic drugs, lifestyles which have been associated with drug-resistant TB elsewhere in the former Soviet Union countries [27,28]. Genotyping studies may be required to identify any linked clusters of HIV-associated MDR-TB. Meta-analysis combining individual patient data from similar studies in other countries could usefully increase the power to detect associations and could add to the current evidence-base on the influence of specific drugs on successful outcome in MDR-TB and XDR-TB patients [18,29].

In conclusion, the findings of this survey have an important bearing on the capacity of the national programme staff and their technical and financing partners to achieve universal access to adequate care for MDR-TB patients [30]. The implications are broad and point towards a need to expand ambulatory and inpatient facilities, the supply of second-line drugs, and safeguarding the quality of drugs [31]. Based on the findings of this study, and in line with Consolidated Action Plan to Prevent and Combat M/XDR-TB in WHO European Region 2011-2015 [32], Uzbekistan is implementing a national action plan to prevent and combat M/XDR-TB. Patient adherence to first-line drug treatment may need strengthening. Early detection of drug-resistant TB and institution of second-line treatment are crucial, as well as other infection control measures. The survey has served to improve the national laboratories' proficiency in undertaking culture and DST (first- and second-line) and LPA. The ongoing expansion in the use of LPA and Xpert MTB/RIF in Uzbekistan is expected to improve access for patient testing. Most cases with rifampicin resistance in this survey also had isoniazid resistance and therefore the positive predictive value of a rifampicin-resistant result for MDR-TB when using new rapid-diagnostic methods such as Xpert MTB/RIF is expected to be high [33].

Since 2011, five new laboratories in the country have acquired the capability to undertake good-quality culture and one more will be able to perform DST reliably shortly. This is expected to increase the patient coverage of DST in Uzbekistan, including the previously untreated TB patients who, as in many other countries, harbour a substantial part of the MDR-TB caseload [34]. If coupled with an efficient electronic system of data capture this would also enable the use of data from routine diagnostic testing for drug resistance surveillance in the near future. Finally, given that international migration is expected to keep increasing in the future, greater efforts to reinforce surveillance and response to the challenge of drug-resistant TB in countries such as Uzbekistan are expected to benefit public health beyond the confines of this country's border.

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Authors' contributions

The survey was undertaken under the supervision of DJ Ulmasova, G Uzakova, H Hoffmann, L Turaev, MN Tillyashayhov, B Kholikulov, A Jalolov, N Muslimova, and J Gadoev. Technical advice on the study design and protocol were provided by W van Gemert, D Falzon, and M Zignol. P du Cros, K Kremer, A Dadu, P de Colombani, O.Telnov, A Slizkiy, M Dara and G Tsogt also contributed to the analysis of the data. The writing of the manuscript was coordinated by D Falzon and all the other co-authors provided substantive contributions to the content and format. All authors agree with the inferences and conclusions drawn.

Conflicts of interest

We declare that we have no conflicts of interest.

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Epidemiological investigation of *Pseudomonas aeruginosa* isolates from a six-year-long hospital outbreak using high-throughput whole genome sequencing

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Although previous bacterial typing methods have been informative about potential relatedness of isolates collected during outbreaks, next-generation sequencing has emerged as a powerful tool to not only look at similarity between isolates, but also put differences into biological context. In this study, we have investigated the whole genome sequence of five *Pseudomonas aeruginosa* isolates collected during a persistent six-year outbreak at Nottingham University Hospitals National Health Service (NHS) Trust – City Campus, United Kingdom. Sequencing, using both Roche 454 and Illumina, reveals that most of these isolates are closely related. Some regions of difference are noted between this cluster of isolates and previously published genome sequences. These include regions containing prophages and prophage remnants such as the serotype-converting bacteriophage D3 and the cytotoxin-converting phage phi CTX. Additionally, single nucleotide polymorphisms (SNPs) between the genomic sequence data reveal key single base differences that have accumulated during the course of this outbreak, giving insight into the evolution of the outbreak strain. Differentiating SNPs were found within a wide variety of genes, including *lasR*, *nrdG*, *tadZ*, and *algB*. These have been generated at a rate estimated to be one SNP every four to five months. In conclusion, we demonstrate that the single base resolution of whole genome sequencing is a powerful tool in analysis of outbreak isolates that can not only show strain similarity, but also evolution over time and potential adaptation through gene sequence changes.

Introduction

Pseudomonas aeruginosa is a Gram-negative bacterium that is widespread in the environment and engages in a wide variety of interactions with eukaryotic host organisms. It is a common opportunistic pathogen in humans, causing a broad range of infections in community and healthcare settings. The most serious manifestations of infection include bacteraemia (particularly in neutropenic patients), pneumonia (particularly in cystic fibrosis patients and critically ill patients), urinary tract infections and wound infections (especially in patients with burn injuries) [1,2]. This organism is intrinsically resistant to many antibiotics and in recent years resistance has emerged to what were previously antimicrobial agents of choice [3,4].

As *P. aeruginosa* is ubiquitous in the environment and also forms part of the resident microbiota of many patients, molecular typing methods are required to identify the sources and routes of transmission within the healthcare setting. Current molecular epidemiological typing systems, such as pulsed-field gel electrophoresis (PFGE) and variable-number tandem repeat (VNTR) analyses, have provided evidence of clonal outbreaks of *P. aeruginosa* affecting clinics or hospitals across the United Kingdom (UK) [5,6]. For example, such approaches have identified several clones (the Liverpool, Midlands 1 and Manchester strains) affecting multiple cystic fibrosis patients in the UK [5-7]. However, these typing methods are of limited resolution when compared to whole genome sequencing. In addition, traditional typing methods fail to provide any insights into the biology of outbreak strains, or show how they might be evolving over time.

A prolonged *P. aeruginosa* outbreak at Nottingham University Hospitals National Health Service (NHS) Trust - City Campus (UH-NHST-CC) that lasted from

2001 to 2007 illustrates some of these issues [8]. Although the outbreak has been brought under control, one to two cases are still seen each year, so it has not been completely eliminated. Here, we have seen a pattern of apparently endemic infection, associated with a single clonal lineage, which was isolated from over 40 patients and also from the hospital environment, as evidenced in previous studies of the isolates [8] and ongoing active surveillance and molecular typing.

The outbreak was first detected when infections caused by five multi-resistant isolates were noted on an 18-bed bone marrow transplant unit at UH-NHST-CC in April 2001. These isolates were resistant to ceftazidime (CTZ), ciprofloxacin (CIP), gentamicin (GEN), piperacillin-tazobactam (TZP), and the carbapenems, but susceptible to amikacin (AMK) and colistin (COL). Surveillance was enhanced, laboratory records were reviewed to identify other cases, and environmental sampling was undertaken. Over the next two years, further isolates from the critical care unit and a new bone marrow transplant unit at the same hospital were identified, initially on the basis of the same antibiogram. As a result of the infection control programmes initiated in response to these outbreaks, infections were identified in 32 haematology patients [8]. Some patients acquired the multi-resistant *P. aeruginosa* without temporal overlap with other positive patients, suggesting an environmental source. Infection control investigations at the time suggested that the outbreak was associated with colonisation of hand washing basins.

Initial molecular epidemiological typing was performed by PFGE using restriction enzyme *Spe*I [9,10] and identified a single outbreak strain among clinical and environmental isolates [8]. Similarity between all isolates was >85% and these formed a single group in cluster analysis, defined as the Nottingham/Trent cluster. In addition, the recommendations of Tenover et al. [10] were followed, which stipulate that isolates belonging to the same strain should differ by a maximum of four fragments. Isolates were additionally typed by random amplification of polymorphic DNA (RAPD) [11] and all demonstrated the same pattern [8]. It was found that in critical care, tracheostomy was the strongest risk factor for acquisition and that the tracheostomy inner tubes were being cleaned in hand wash basins that were found to be contaminated. The correct fitting of waste trap heaters, the removal of one persistently colonised sink, enhanced surveillance, and a review of equipment and sink cleaning, particularly in the case of tracheostomy equipment helped to bring the outbreak under control. In clinical haematology, oral colistin was introduced for selective decontamination of the gut during neutropenia and use of ciprofloxacin, to which the strain is resistant, was reduced for neutropenic prophylaxis.

Whole genome sequencing, facilitated by the advent of high-throughput approaches, brings the promise of single-base-pair resolution between isolates, making it the ultimate molecular typing method for bacteria. Several recent studies have shown that analysis of single nucleotide polymorphisms (SNPs) in bacterial genomes provides a means of determining relatedness between epidemiologically linked isolates and tracking bacterial evolution over periods of months to years [12-16]. In particular, several studies have tracked the genetic changes that emerge during long-term colonisation of cystic fibrosis patients [17,18]. In addition, genome sequencing provides a strain-specific list of protein-coding genes, which, through comparisons with the genes sets of other strains from the same species can provide insights into the unique biology of an epidemic strain. We therefore applied high-throughput sequencing to five isolates from the UH-NHST-CC hospital outbreak to determine whether genome sequencing can inform our understanding of the epidemiology and biology of a hospital pathogen in this setting.

Table 1

Selection for sequencing of *Pseudomonas aeruginosa* isolates originating from an outbreak in Nottingham that occurred between 2001 and 2007, United Kingdom

Isolate	Isolation date	Specimen	Sequencing platform
PANOTK11	Jun 2001	Swab from line site	Illumina
PANOT101	Jan 2002	Urine	Illumina
PANOT106	Jan 2003	Blood	454 and Illumina
PANOT340	Apr 2005	Swab from line site	Illumina
PANOT738	Dec 2007	Faeces	Illumina

NHS: National Health Service.

Methods

Microbiology

Isolates of *P. aeruginosa* were collected from the microbiological specimens of patients cared for at the Queen's University Medical Centre over a six-year period from January, 2002 to December, 2007. Isolates were frozen on beads and stored at -80°C. *P. aeruginosa* isolates were sent to the Health Protection England's (HPE) Laboratory of HealthCare Associated Infection in London for analysis by PFGE. Four isolates from different time points in the outbreak were selected for genome sequencing in this study on the basis of PFGE and RAPD results, as well as an additional isolate from June 2001 (Table 1). This additional isolate was identified as part of the look-back exercise initiated after the first outbreak; its PFGE profile was established by the HPE. The antimicrobial susceptibilities of isolates were tested using the disk diffusion method against AMK, aztreonam (AZT), CTZ, COL, CIP, GEN, meropenem (MER), tobramycin (TOB) and TZP and according to

British Society for Antimicrobial Chemotherapy (BSAC) guidelines [19].

Isolation of genomic DNA and high-throughput sequencing

Genomic DNA was obtained from colony-purified *P. aeruginosa*, using the DNeasy DNA extraction kit according to the manufacturer's instructions (Qiagen). DNA from isolate PANOT106 was sequenced using whole genome shotgun and 2.5-kilobase paired-end 454 protocols in accordance with the manufacturer's instructions (Roche) at the University of Liverpool's Centre for Genomic Research. Isolates PANOT106, PANOTK11, PANOT101, PANOT340, and PANOT738 were sequenced one sample per lane using the Illumina GA2, running 35 cycles with paired-end libraries (~250 base-pair insert size). Sequencing on the Illumina GA2 platform was carried out at the sequencing centre of the University of Washington at St. Louis.

Genome assembly

Roche 454 reads for isolate PANOT106 were assembled de *novo* with Newbler v2.3 (Roche) to create a reference assembly, using the default settings with –g enabled to produce contig graphs for use later in the variant detection pipeline. Nesoni 0.51 (Victorian Bioinformatics Consortium) was used on the Illumina reads for PANOT106 to correct errors in the reference assembly, particularly short indels in regions with low depth of coverage or in homopolymeric tracts. Nesoni parameters were: depth 10; purity 0.8; ambiguity-codes 1; fidelity 1.0; monogamous 1; trim 0; max-pair-sep 250; only-pairs 1; suffix1 /1; suffix2 /2; same-dir 0; circular 0; savehits 0. Bowtie v0.12.0 [20] was used to align the PANOT106 Illumina reads to complete *P. aeruginosa* genomes available in GenBank, using parameters --solexa -quals -S -m 1 -p 4. The progressive Mauve component of Mauve (version 2.3.1) was used to map the PANOT106 reference assembly to the genome sequence of *P. aeruginosa* PAO1.

Illumina sequences for isolates PANOTK11, PANOT101, PANOT340, and PANOT738 were assembled using SOAPdenovo 1.03 (http://soap.genomics.org.cn/soapdenovo.html). Assemblies were individually annotated using the xBASE annotation pipeline [21-26].

Creation of a well-validated single nucleotide polymorphism (SNP) set

A series of filters were applied to the sequence data to create a set of high-confidence SNP variants. Bowtie v0.12.0 was used to align the Illumina data for each of the five sequenced strains to the PANOT106 reference assembly and variants extracted using samtools with parameters –cv –N *n* to create set ‹VARIANTS›. The value of *n* was set to the number of isolates in the sample.

Putative variants were filtered with samtools.pl varFilter to create set ‹FILTER1›. varFilter was run with the following parameters: -Q 25 -d 10 -D 100 -G 25 -w 10 -W 10 -N 2 -l 30, equating to the following filtering options: Minimum root mean square mapping quality for SNPs, 25; Minimum root mean square mapping quality for gaps, 10; Minimum read depth, 10; Maximum read depth, 90; Minimum indel score for nearby SNP filtering, 25; SNP within INT bp around a gap to be filtered, 10; Window size for filtering dense SNPs, 10; Max number of SNPs in a window, 2; Window size for filtering adjacent gaps, 30.

These variant calls were further filtered to a set with SNP quality of 30 (phred-scale) to create set ‹FILTER2›. The position of each variant call was related to the *P. aeruginosa* strain PAO1 genome sequence by reference to the Mauve alignment. Variant calls with no corresponding alignment in strain PAO1 were discarded, creating set ‹FILTER3›. Variant calls which occurred in contigs in the reference assembly which were not included in the scaffold assembly and thus were likely to be repetitive were further excluded from analysis, resulting in ‹FILTER4›. Variant calls which occurred in contigs which had coverage depth greater or less than two standard deviations from the mean were excluded, resulting in ‹FILTER5›. Finally, variant calls with a variant frequency of less than 0.9 were discarded. Variant frequency was determined by dividing the alignment depth by the number of reads supporting the variant call.

Analysis of gene content

Protein-coding sequences (CDSs) in the PANOT106 assembly were identified using Gene Locator and Interpolated Markov ModelER (GLIMMER) [23]. Protein Basic Local Alignment Search Tool (BLASTP) [21] was used to compare the set of CDSs in PANOT106 with the predicted protein repertoires of other completed *P. aeruginosa* genome sequences. Regions of difference (RODs) between the PANOT106 and the known *P. aeruginosa* pan-genome (as defined by complete genomes in GenBank) were identified on the basis of the following criteria: the ROD must contain ≥ 10 strain-specific CDSs, which have no orthologues in other genomes (as defined by mutual best-hit analysis), with the ROD interrupted by no more than five genes already present in the pan-genome. Orthologues were computed using OrthoMCL v2.0 with default parameters [27]. Singletons (coding sequences which do not have orthologues in other outbreak isolates) were verified by translated nucleotide databases BLAST (TBLASTN) searches against the original sequence. Additional information with regards to the annotated features made use of the *Pseudomonas* Genome Database [28].

Results

Assembly of the genome sequence data

Using the Roche 454 next-generation genome sequencing platform, 6,720,954 bases were generated from the DNA extracted from isolate PANOT106. Reads were assembled, using Newbler, into 1,059 contigs, which were further assembled into 19 scaffolds using

Table 2

Illumina sequencing results for *Pseudomonas aeruginosa* isolates originating from an outbreak in Nottingham that occurred between 2001 and 2007, United Kingdom

Isolate	Total reads	Reads per pair	Bases	Coverage	Scaffolds	Maximum scaffold size	Bases in scaffolds	Average scaffold size	N50 scaffold size	N90 scaffold size
PANOTK11	11,936,530	5,968,265	417,778,550	~70X	393	168,419	6,857,254	11,024	41,132	9,556
PANOT101	15,007,136	7,503,568	525,249,760	~88X	189	288,005	6,823,034	17,494	110,879	25,874
PANOT106	15,392,542	7,696,271	538,738,970	~90X	202	414,841	6,823,529	18,196	81,409	20,375
PANOT340	10,684,280	5,342,140	373,949,800	~62X	756	161,292	6,821,477	6,363	20,384	3,728
PANOT738	14,983,686	7,491,843	524,429,010	~87X	294	225,595	6,818,630	14,001	51,282	12,127

NHS: National Health Service.

paired-end sequence data to determine the relative positions and distances of contigs. The N50 of the scaffolds is 825,048 and the average paired end distance is 2,365 bases, with a standard deviation of 591 bases.

The next-generation sequencing data generated on the Illumina platform for isolates PANOT106, PANOTK11, PANOT101, PANOT340, and PANOT738, is summarised in Table 2. Coverage with the 35 base Illumina reads ranged from 62X to 90X. The data for isolate PANOT106 was used to correct the assembly of this genome sequence based on the 454 data. This resulted in 283 total edits to the original assembled sequence.

Antibiogram differences

The only detected difference in antibiotic resistance amongst the five isolates was AMK resistance in PANOTK11 (data not shown). Resistance to aminoglycosides is typically associated with aminoglycoside-modifying enzymes. Each of the outbreak strains carry identical aminoglycoside 3'-phosphotransferase type IIb coding sequences homologous to the *Pseudomonas* strain PAO1 CDS with accession PA4119. Additionally there were no SNPs detected upstream to suggest a change in promoter in PANOTK11. The alternative resistance-conferring aminoglycoside 4'-O-adenylyltransferase IIb [29] was not found in any of the outbreak strains. We therefore conclude that AMK resistance in PANOTK11 is probably due to impermeability or efflux, although even if true, this fails to explain why resistance to other aminoglycosides was unaffected [30].

Novel sequences within the genomes of the outbreak isolates

There are six large regions of difference between PANOT106 and the *P. aeruginosa* pan-genome (Figure panel A-F), five of which appear to be prophages or prophage remnants. In particular, PANOT106 contains a region that resembles the serotype-converting *P. aeruginosa* bacteriophage D3 and a region that encodes homologues of phage structural proteins from phi CTX, a cytotoxin-converting phage of *P. aeruginosa*. The only non-prophage region of difference contains copper- and arsenic-resistance genes and is homologous to a similar region in the genome of *Stenotrophomonas* sp. SKA14.

PANOTK11 has a 48 kb sequence that is not present in the other strains sequenced here (Figure panel G). This region was assembled into a single contig and was annotated to contain 24 CDSs. Although this region has no homologue in any of the other strains sequenced here, it is homologous to sequence data from isolate PACS171b [31], which was isolated from the oropharynx of a nine month-old cystic fibrosis patient [32]. Of the 48 kb, all but 13 kb share similarity with isolate PACS171b; these 35 kb mostly contain hypothetical genes and a putative restriction-modification system and putative sigma-54 transcriptional regulator. Within the 13 kb region that is different from isolate PACS171b, isolate PANOTK11 contains a member of the DEAD box helicase family, an OLD family endonuclease, and a hypothetical gene.

No novel sequences were found within the genomes of PANOT101, PANOT340, and PANOT738, in comparison with the *P. aeruginosa* pan-genome.

Flagellum operon

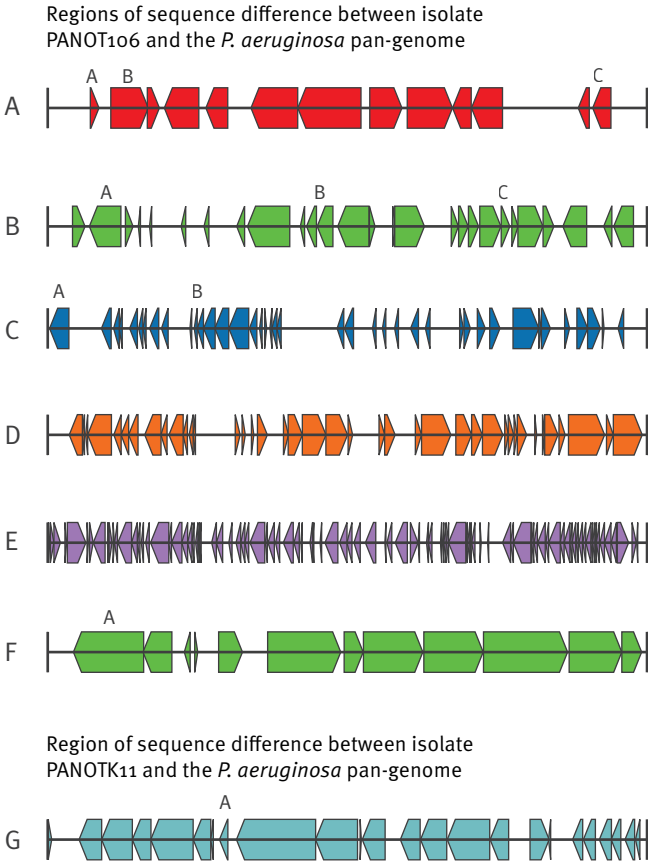
All five of the outbreak strains are missing a portion of the flagellum operon compared to that present in *P. aeruginosa* strains PAO1 and LESB58. The corresponding CDS PA1088, encoding flagellar glycosyl transferase FgtA is not present in the outbreak isolates sequenced here. The *fliC* (PA1092) gene and CDSs PA1092–PA1096 are present, although these are divergent in their nucleotide sequences.

Genome-wide single nucleotide polymorphism (SNP) analysis of outbreak strains

The PANOT106 sequence, assembled from Illumina and 454 sequence data, aligns well with the *P. aeruginosa* strains PAO1 (95.3% sequence identity),

Figure

Regions of sequence difference between Nottingham *Pseudomonas aeruginosa* isolates and the *P. aeruginosa* pan-genome



Arrowed blocks show annotated coding regions, indicating their direction of transcription.

Panel A. A 17.2 kb prophage region containing a chromate resistance protein (A), chromate transporter (B), and phage structural proteins (C).

Panel B. A 29.4 kb copper/arsenate resistance region containing a heavy metal Resistance-Nodulation-cell Division (RND) efflux outer membrane protein (A), a copper resistance operon (B), and arsenate resistance genes (C).

Panel C. A 32.1 kb lambda-like prophage region containing a phage integrase (A) and alginate biosynthesis regulatory protein (B).

Panel D. A 35.3 kb prophage region.

Panel E. A Phi-CTX-like prophage region.

Panel F. A 21.4 kb serine protease containing region of difference containing subtilisin-related serine protease (A).

Panel G. A 47.9 kb region of difference from PANOTK11 containing a tellurium resistance protein (A).

LESB58 (92.5%), and PA14 (92.4%) genome sequences and less well with PA7 (77.8%). Comparisons of the Ilumina sequences from isolates PANOTK11, PANOT101, PANOT340, and PANOT738 with the PANOT106 assembly generated a large number of variant calls. Several hundred variants were discarded during the filtering of variants to identify high-quality informative SNPs that vary between the outbreak isolates and which are associated with 37 polymorphic loci (Table 3). Twenty-four of the SNPs result in non-synonymous changes to the associated encoded amino acid in the PAO1-annotated coding region (Table 4). This high frequency of non-synonymous changes (24 of 37) suggests that some of the SNPs represent adaptive changes.

Nineteen of the SNPs separate PANOTK11 from the other four UH-NHST-CC Nottingham isolates (Table 3). None of these nineteen SNPs are found in any of the other four outbreak isolates, which all share greater similarity to the reference strain PAO1 at these SNP loci. These SNPs in this isolate, the 48 kb region present only in this isolate, and the AMK resistance of this isolate indicate that PANOTK11, despite being identified as related to the other outbreak isolates as part of the Nottingham/Trent cluster via PFGE and RAPD, is not part of the same outbreak and cannot represent the index case. Despite the earlier typing results, PANOTK11 is a phylogenetic outlier when compared to other outbreak isolates on the genomic level. Interestingly, one of these PANOTK11-specific SNPs (PNSNP12) maps to *lasR* (Table 4), encoding a transcriptional regulator that responds to a homoserine lactone signal to activate expression of *P. aeruginosa* virulence factors through quorum sensing. Mutations in *LasR* have been reported in several contexts, including adaptation to the airways of cystic fibrosis patients [33]. PNSNP12 does not appear to be within the helix-turn-helix DNA binding domain of the encoded protein according to the available sequence and functional information in UniProt [35], and to predictions by the helixturnhelix (HTH) software tool [34] but SNP mutations in other *LasR* regions, including the ligand binding domain, have been reported previously [36]. Another of the PANOTK11 SNPs (PNSNP34) maps to *algB* (Table 4), encoding an NtrC-like response regulator, which controls alginate biosynthesis in mucoid *P. aeruginosa* [37]. In this case the SNP falls within the helix-turn-helix motif predicted by HTH [34] and described previously [38]. This SNP may therefore impact upon the virulence of PANOTK11 and differences in alginate biosynthesis should be explored.

PANOT101 and PANOT106 were isolated a year apart (2002 and 2003, respectively) and share many of the same non-synonymous SNPs, apart from five identified here (Table 3). PNSNP10, within a sensor/response regulator hybrid (PA0928), and PNSNP25, within *nrdG*, in PANOT106 are also shared with isolates PANOT340 and PANOT738 (Tables 3 and 4). PNSNP11 and PNSNP13 in a hypothetic protein (PA0938) and two-component sensor (PA1458), respectively, are only found in PANOT106 in this study. Likewise, PNSNP26 in PANOT101 (Table 3) is only found within this isolate in this study. PNSNP26 is within *speC* encoding ornithine decarboxylase (PA4519; Table 4) part of the biosynthetic pathway to produce putrescine, required for growth, but loss of function of this protein can be compensated through an alternative putrescine biosynthesis pathway using *speA* [39].

Table 3

Comparison of well-validated single nucleotide polymorphisms in the *Pseudomonas aeruginosa* Nottingham outbreak isolates

SNP ID	*P. aeruginosa* Nottingham outbreak isolates				
	PANOT101	PANOT106	PANOT340	PANOT738	PANOTK11
PNSNP1	G	G	G	G	A
PNSNP2	T	T	G	G	T
PNSNP3	C	C	C	A	C
PNSNP4	T	T	T	T	C
PNSNP5	G	G	T	T	G
PNSNP6	A	A	A	A	G
PNSNP7	A	A	A	A	T
PNSNP8	T	T	T	T	C
PNSNP9	A	A	A	A	G
PNSNP10	T	G	G	G	T
PNSNP11	A	T	A	A	A
PNSNP12	G	G	G	G	A
PNSNP13	C	T	C	C	C
PNSNP14	G	G	G	G	C
PNSNP15	T	T	T	T	A
PNSNP16	G	G	G	T	G
PNSNP17	C	C	C	C	T
PNSNP18	C	C	C	A	C
PNSNP19	C	C	T	T	C
PNSNP20	A	A	A	A	C
PNSNP21	C	C	C	C	G
PNSNP22	C	C	C	A	C
PNSNP23	A	A	G	G	A
PNSNP24	A	A	A	A	C
PNSNP25	T	G	G	G	T
PNSNP26	A	C	C	C	C
PNSNP27	G	G	G	T	G
PNSNP28	A	A	A	A	G
PNSNP29	T	T	T	T	C
PNSNP30	C	C	C	C	T
PNSNP31	G	G	G	A	G
PNSNP32	A	A	C	C	A
PNSNP33	A	A	A	C	A
PNSNP34	G	G	G	G	C
PNSNP35	C	C	C	A	C
PNSNP36	G	G	G	G	T
PNSNP37	T	T	T	T	G

ID: identity; SNP: single nucleotide polymorphism.

The two subsequent strains, PANOT340 and PANOT738, isolated in 2005 and 2007, respectively, show a progressive increase in the number of SNPs relative to PANOT106. These three isolates share two non-synonymous SNPs (Table 3), one, PNSNP10, within a sensor/response regulator hybrid (PA0928, *gacS*) and one, PNSNP25, in a class III (anaerobic) ribonucleoside-triphosphate reductase activating protein gene (PA1919, *nrdG*). Five shared non-synonymous SNPs (PNSNP2, PNSNP5, PNSNP19, PNSNP23, and PNSNP32) are present in isolates PANOT340 and PANOT738, but not in the other outbreak isolates. In isolate PANOT738, an additional eight SNPs are present, three leading to non-synonymous changes (Table 3) and five in intergenic regions. This progressive accumulation of SNPs is consistent with an ancestor-descendant relationship. The acquisition of five SNPs in two and a third years (5 SNPs in 28 months, or 5.6 months per SNP) and a further eight SNPs in two and two third years (8 SNPs in 32 months, or 4 months per SNP), with 13 over a span of five years from January 2003 to December 2007 (13 SNPs in 60 months, 4.6 months per SNP) suggests that a new SNP is acquired in this lineage on average every four to five months.

The five non-synonymous SNPs in common between isolates PANOT340 and PANOT738 include PNSNP32 which is associated with a type II citrate synthase (PA1580, *gltA*), PNSNP2 in a hypothetical protein that may be a thioesterase (PA3130), PNSNP5 in a putative methylated-DNA--protein-cysteine methyltransferase (PA3596), PNSNP23 in a probable two-component response regulator (PA5364), and PNSNP19 in an ATP-dependent protease peptidase subunit of heat shock protein HslV (PA5053, *hslV*). The small form of the citrate synthase protein encoded by *gltA* is involved in the Krebs cycle and is expressed in excess during stationary phase growth [40]. Whether PNSNP32 has some bearing on the stationary phase growth rate of isolates PANOT340 and PANOT738 in comparison with isolate PANOT106 requires further investigation, as this may impact upon survival of the bacteria in the hospital setting. PNSNP5 is within a CDS potentially encoding a methylated-DNA--protein-cysteine methyltransferase, which is involved in the repair of alkylated DNA (EC 2.1.1.63). Sensitivity testing for alkylating agents of the outbreak isolates may be warranted in light of the PNSNP5 data.

The remaining eight SNPs present only in isolate PANOT738 are associated with non-synonymous changes in three different CDSs and five intergenic regions. The three SNPs in CDSs are PNSNP33 found within a putative class III pyridoxal phosphate-dependent aminotransferase (PA0530), PNSNP27 in *tadZ* (PA4303), and PNSNP16 in a putative transcriptional regulator (PA2846). The CDS for *tadZ* is within the widespread colonisation island (WCI) encoding tight adherence pili. The genes of the WCI are essential for biofilm formation and colonisation [41], which are important virulence factors in *P. aeruginosa*. The PNSNP27 within *tadZ* may therefore influence the success of isolate PANOT738 in forming biofilms and colonising the host, medical devices, or even medical surfaces such as the persistently colonised sink. Isolate PANOT738 also contains PNSNP16 that is within PA2846, which is one of several predicted transcriptional (PA1430)

Table 4

Non-synonymous single nucleotide polymorphisms in the genome sequence data of *Pseudomonas aeruginosa* isolates from the outbreak in Nottingham

SNP ID[a]	Isolate SNP[b]	PAO1[c]	Position in PAO1[d]	PAO1 locus[e]	PAO1 CDS annotation	PAO1 amino acid	SNP amino acid
PNSNP2	(C)	A	3512351	PA3130	Hypothetical protein	V	G
PNSNP4	(G)	A	4128733	PA3687	Phosphoenolpyruvate carboxylase	W	R
PNSNP5	(A)	C	4032021	PA3596	Putative methylated-DNA--protein-cysteine methyltransferase	C	F
PNSNP7	(A)	T	4000131	PA3568	Putative acetyl-coa synthetase	I	F
PNSNP9	G	A	827118	PA0758	Hypothetical protein	I	T
PNSNP10	G	T	1014510	PA0928	Sensor/response regulator hybrid	T	P
PNSNP11	T	A	1028593	PA0938	Hypothetical protein	Q	L
PNSNP12	A	G	1558547	PA1430	Transcriptional regulator LasR	G	D
PNSNP13	T	C	1588334	PA1458	Two-component sensor	R	C
PNSNP14	C	G	1590208	PA1459	Chemotaxis-specific methylesterase	G	R
PNSNP15	A	T	1635647	PA1505	Molybdenum cofactor biosynthesis protein A	L	Q
PNSNP16	(A)	C	3201052	PA2846	Putative transcriptional regulator	L	I
PNSNP17	(A)	G	3007141	PA2656	Putative two-component sensor	L	F
PNSNP19	(A)	G	5692646	PA5053	ATP-dependent protease peptidase subunit	G	D
PNSNP23	(C)	T	6031991	PA5364	Putative two-component response regulator	N	S
PNSNP25	G	T	2093908	PA1919	Ribonucleoside-triphosphate reductase activating protein, NrdG	T	P
PNSNP26	A	C	5060237	PA4519	Ornithine decarboxylase	G	C
PNSNP27	T	G	4827417	PA4303	TadZ	P	Q
PNSNP29	T	C	5173073	PA4615	Putative oxidoreductase	E	K
PNSNP32	(G)	T	1719347	PA1580	Type II citrate synthase	D	A
PNSNP33	(G)	T	589009	PA0530	Putative class III pyridoxal phosphate-dependent aminotransferase	L	R
PNSNP34	(G)	C	6174644	PA5483	Two-component response regulator AlgB	L	V
PNSNP36	T	G	2686727	PA2402	Peptide synthase	S	Stop
PNSNP37	G	T	2686790	PA2402	Peptide synthase	D	A

CDS: protein coding sequence; ID: identity; SNP: single nucleotide polymorphism; Stop: termination codon.

[a] SNP ID from Table 3.

[b] SNP from one of the isolates as listed in Table 3. Reverse complementary bases, in parenthesis, have been entered where the PAO1 strand is negative relative to the data in Table 3.

[c] Base at this SNP location within the PAO1 genome sequence.

[d] Position of the SNP within the PAO1 genome sequence.

[e] CDS locus identifier from the PAO1 genome sequence.

and response regulators (PA0928, PA1458, PA2656, PA5364, PA5483) that contain SNPs in these outbreak isolates, suggesting that differences in regulation may play a role in the phenotypes observed.

Discussion

Here, we have shown that genome sequence data obtained with high-throughput sequencing technologies can provide novel insights into the epidemiology and biology of hospital isolates of *P. aeruginosa*. One isolate, PANOTK11, was determined to be an outlier and not part of the same outbreak lineage as the other four isolates. This is based on genomic level sequence differences; previous typing with PFGE and RAPD placed all five of these isolates into a single lineage.

Amongst the related isolates, we have demonstrated that *P. aeruginosa* lineages from a hospital setting contain a sufficient number of SNPs to allow the construction of an epidemiological narrative — an important benchmarking exercise that lays the foundations for future investigations into the fine-grained genomic epidemiology of this troublesome pathogen.

In addition, genome sequencing has highlighted the existence of strain-specific gene clusters that might underpin the tenacious survival of these strains within our hospital over many years. Several SNPs which may confer phenotypic differences between the isolates have been identified and bear further investigation, particularly as some may play a role in survival of isolates in the hospital. These include SNPs within PA1580 that could influence stationary phase growth rate and impact on the survival of the bacteria in the hospital setting, within PA3596 that could influence the sensitivity to alkylating agents, and within PA4303 that may be important for biofilm formation.

This study illustrates the potential of the first generation of high-throughput sequencing platforms in the investigation of hospital outbreaks. Relentless improvements in cost and performance (particularly those delivered recently by bench top sequencing [42]) as well as data analysis software, are taking these technologies ever closer to the clinical microbiology laboratories. With a price tag now comparable to the cost of traditional molecular typing, bacterial genome sequencing is now poised to impact on clinical practice.

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Authors' contributions

DAA, MJP, NJL, GW conceived of and designed the study; DAA, LAS, KL, TCB carried out clinical, epidemiological and microbiological analyses; GW supervised genome sequencing; NJL, LAF and LAS performed bioinformatics analyses; MJP, DAA, NJL, LAS wrote the paper.

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