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Antimicrobial susceptibility and emerging resistance determinants (bla_{CTX-M}, *rmtB*, *fosA3*) in clinical isolates from urinary tract infections in the Bolivian Chaco

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SUMMARY

Background: Bolivia is among the lowest-resourced South American countries, with very few data available on antibiotic resistance in bacterial pathogens. The phenotypic and molecular characterization of bacterial isolates responsible for urinary tract infections (UTIs) in the Bolivian Chaco are reported here.
Methods: All clinical isolates from UTIs collected in the Hospital Basico Villa Montes between June 2010 and January 2014 were analyzed ($N = 213$). Characterization included susceptibility testing, extended-spectrum beta-lactamase (ESBL) detection, identification of relevant resistance determinants (e.g., CTX-M-type ESBLs, 16S rRNA methyltransferases, glutathione S-transferases), and genotyping of CTX-M producers.
Results: Very high resistance rates were observed. Overall, the lowest susceptibility was observed for trimethoprim–sulphamethoxazole, tetracycline, nalidixic acid, amoxicillin–clavulanic acid, ciprofloxacin, and gentamicin. Of *E. coli* and *K. pneumoniae*, 11.6% were ESBL producers. Resistance to nitrofurantoin, amikacin, and fosfomycin remained low, and susceptibility to carbapenems was fully preserved. CTX-M-15 was the dominant CTX-M variant. Four *E. coli* ST131 (two being H30-Rx) were identified. Of note, isolates harbouring *rmtB* and *fosA3* were detected.
Conclusions: Bolivia is not an exception to the very high resistance burden affecting many South American countries. Optimization of alternative approaches to monitor local antibiotic resistance trends in resource-limited settings is strongly encouraged to support the implementation of effective empiric treatment guidelines.

1. Introduction

South America has long been documented as being affected by high antibiotic resistance rates.[1–5] Complex political and socio-economic factors account for this burden, and the precise quantification at the local, national, and supranational level deserves further attention.[1–4] Bolivia is one of the lowest-resourced countries of South America, and very few data on the rates and molecular epidemiology of antibiotic resistance in bacterial pathogens have been reported from this area.[1–3,5]

Since the late 1990s, cooperation and research activities addressing the phenomenon of antibiotic resistance in the Bolivian Chaco region have been performed by the present investigators, in collaboration with the Bolivian Ministry of Health.[6–13] The healthcare system of this region, which includes many rural areas and native villages, essentially relies upon small hospitals with

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limited access to clinical microbiology facilities, which prevents any systematic collection of antimicrobial susceptibility data from the routine microbiological analysis of clinical specimens. By using commensal *Escherichia coli* as an indicator for the dissemination of antibiotic resistance in enterobacteria, very high resistance rates to old antibiotics (i.e., ampicillin, trimethoprim–sulphamethoxazole, tetracycline, chloramphenicol, and nalidixic acid) and alarmingly increasing trends of resistance to newer drugs (i.e., expanded-spectrum cephalosporins and fluoroquinolones) have been observed over the last two decades.[8–13] Of note, CTX-M-type extended-spectrum beta-lactamase (ESBL) determinants were first detected in this area in the early 2000s and thereafter underwent rapid dissemination, with an evolution of the dominant CTX-M groups mirroring that observed in other South American countries (i.e., initial dissemination of CTX-M-2, subsequently replaced by CTX-M-1 and CTX-M-9 groups).[1–3,8,14–16]

In this study, data on antimicrobial susceptibility and resistance determinants of bacterial pathogens responsible for urinary tract infections (UTIs) in the Bolivian Chaco region are reported. These data were obtained by analyzing clinical isolates from UTIs collected in the laboratory of the Hospital Basico Villa Montes (one of the first clinical microbiology laboratories implemented in that region) during the first 3 years of activity.

2. Methods

2.1. Bacterial isolates

A total of 213 non-replicate clinical isolates from UTIs were included in the study. They represented all of the isolates from positive urine cultures of patients with a clinical diagnosis of UTI, submitted to the clinical microbiology laboratory of the Hospital Basico Villa Montes (Villa Montes, Tarija Department, Bolivia) since its activation in June 2010, up to January 2014. Clinical isolates from both inpatients and outpatients were included. The isolates were stored in Amies transport medium (Oxoid, Milan, Italy) at 4 °C before being transferred to Italy.

2.2. Bacterial identification and in vitro antibiotic susceptibility testing

Bacterial identification was performed by matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry (Vitek MS, bioMérieux Inc., Marcy l'Etoile, France). Antibiotic susceptibility was tested by disk diffusion method in accordance with Clinical and Laboratory Standards Institute (CLSI) guidelines.[17,18] As the CLSI does not provide interpretative criteria for fosfomycin and *Enterobacteriaceae* other than *E. coli*, results for the former species were interpreted using *E. coli* breakpoints (i.e., susceptible when the inhibition zone diameter is $\geq$16 mm),[18] as also reported in other studies (Endimiani et al.[19] and references therein). Screening and confirmatory tests for ESBL detection were carried out according to CLSI standards.[18] The production of AmpC-like enzymes was suspected on the basis of resistance to cephamycins and inhibition by 3-aminophenylboronic acid.[20] In AmpC producers, the presence of ESBLs was also investigated by modified CLSI confirmatory test, as recently proposed by Poulou et al.[21]Fosfomycin non-susceptible isolates were tested for the production of glutathione S-transferases using the disk potentiation test recently developed by Nakamura et al. (based on the inhibition of glutathione S-transferases by sodium phosphonoformate).[22]

2.3. Molecular analysis techniques

The detection and characterization of $bla_{\text{CTX-M}}$ and bla_{AmpC}-like beta-lactamase genes was performed using a PCR sequencing approach, as described previously.[8,23] PCR amplification was also used for the detection and characterization of the fosfomycin resistance genes *fosA*, *fosA3*, and *fosC2* (encoding glutathione S-transferases),[24] the aminoglycoside resistance genes *armA*, *npmA*, *rmtA*, *rmtB*, *rmtC*, *rmtD*, *rmtE*, *rmtF*, *rmtG*, and *rmtH* (encoding 16S rRNA methyltransferases), and *aac(6′)Ib* (encoding aminoglycoside acetyltransferases).[25,26] The determination of *E. coli* phylogenetic groups was carried out by multiplex PCR.[27] Established PCR-based methods were used to define the *E. coli* clone B2-O25b-ST131 and its subclones H30 and H30-Rx.[28–30]

2.4. Statistical analysis

Statistical differences were determined by Chi-square test (with Yates' correction) and the unpaired *t*-test, using GraphPad Prism for Windows, version 5.0 (GraphPad Software, San Diego, CA, USA).

3. Results and discussion

3.1. Patient characteristics and aetiology of UTIs

A total of 213 clinical isolates considered responsible for UTIs were collected during the study period (June 2010 to January 2014). The study started upon activation of the clinical microbiology facility. A very low number of urine samples were processed during the first 2 years of activity (due to initial difficulties in implementing the facility), while an increasing trend was observed after 2012. As a consequence, the distribution of clinical isolates over time was as follows: 2010 (7 months), $n = 10$; 2011, $n = 12$; 2012, $n = 73$; 2013, $n = 105$; 2014 (1 month), $n = 13$.

Of the 213 clinical isolates, 71 were from inpatients and 140 were from outpatients; the origin was unknown for two isolates. The overall male to female patient ratio was 40:171, with the sex of two patients unknown. Patients ranged in age from 2 months to 95 years (mean age 44 years, median age 45 years), with the age of 16 patients unknown. Inpatient and outpatient populations differed in age distribution (mean age 54 vs. 39 years, median age 55 vs. 37 years; $p = 0.0003$), while no significant difference was observed in the male to female ratio (12:59 vs. 28:111; $p = 0.57$).

Of the 213 clinical isolates, 209 (98.1%) were *Enterobacteriaceae*, three were *Pseudomonas aeruginosa* (1.4%), and one was a *Staphylococcus saprophyticus* (0.5%) (Table 1). *E. coli* represented the dominant species (79.8%), followed by *Klebsiella pneumoniae* (8.9%) (Table 1), with no differences observed between inpatients and outpatients (data not shown).

3.2. Antibiotic susceptibility

Overall, the UTI isolates collected from the Bolivian Chaco exhibited high rates of antibiotic resistance (Table 2; **Supplementary Material** Table S1).

Table 1
Aetiology of urinary tract infections in the Bolivian Chaco (2010–2014)

Species	No. of isolates	%
Escherichia coli	170	79.8
Klebsiella pneumoniae	19	8.9
Citrobacter spp	6	2.8
Enterobacter spp	5	2.3
Proteus spp	5	2.3
Morganella morganii	3	1.4
Pseudomonas aeruginosa	3	1.4
Providencia rettgeri	1	0.5
Staphylococcus saprophyticus	1	0.5

Table 2
Antibiotic susceptibility rates (%) of *Escherichia coli* and *Klebsiella pneumoniae* urinary isolates

Drug[a]	*E. coli* (*n* = 170)	*K. pneumoniae* (*n* = 19)	Total (*n* = 189)
AMC	56.5	47.3	55.6
Cefotaxime	89.4	68.4	87.3
Ceftazidime	92.3	73.7	90.5
Meropenem	100	100	100
SXT	26.5	52.6	28.6
Nalidixic acid	51.2	63.2	52.4
Ciprofloxacin	61.8	68.4	62.4
Gentamicin	72.4	68.4	72.0
Amikacin	95.3	89.5	94.7
Nitrofurantoin	91.8	31.6	85.7
Fosfomycin	98.2	94.7	97.9
Tetracycline	47.1	57.9	47.6

[a] AMC, amoxicillin–clavulanic acid; SXT, trimethoprim–sulphamethoxazole.

Considering *E. coli* and *K. pneumoniae*, which were by far the most prevalent pathogens, the most affected drugs were trimethoprim–sulphamethoxazole, tetracycline, nalidixic acid, amoxicillin–clavulanic acid, and ciprofloxacin (Table 2). Expanded-spectrum cephalosporins remained active against the majority of isolates (87.3% and 90.5% susceptibility to cefotaxime and ceftazidime, respectively) (Table 2). Of the 24 isolates resistant to cefotaxime, 22 were identified as ESBL producers (11.6% of all *E. coli* and *K. pneumoniae* isolates). The remaining two isolates showed a resistance phenotype suggestive of the production of AmpC-like enzymes. Expanded-spectrum cephalosporin-resistant isolates were significantly more prevalent among *K. pneumoniae* than *E. coli* isolates (31.6% vs. 10.6%; $p = 0.009$). Overall, the most active drugs were meropenem, fosfomycin, amikacin, and nitrofurantoin (the latter only for *E. coli*) (Table 2). *K. pneumoniae* isolates exhibited very low susceptibility to nitrofurantoin, in accordance with previous reports.[31] The few non-enterobacterial pathogens showed susceptible phenotypes typical of wild-type strains, except for resistance to ciprofloxacin exhibited by one of the three *P. aeruginosa* isolates (data not shown). Of note, no significant difference in terms of antibiotic resistance rates and prevalence of ESBL phenotype was observed between clinical isolates from inpatients and outpatients (data not shown), while an increasing trend (statistically significant for expanded-spectrum cephalosporins, quinolones, and nitrofurantoin) was observed with age (**Supplementary Material** Table S2).

The high resistance rates observed in clinical isolates from UTIs from the Bolivian Chaco were overall consistent with those reported from other Latin American countries,[1–4] and with data collected in Bolivia by the Pan-American Health Organization (PAHO).[5,32]

One limitation of the present study, which might have introduced a bias towards an overestimation of resistance rates, is that in Bolivia microbiological diagnosis represents an extra cost for the patient, and urine specimens for culture are rarely requested by physicians for the diagnosis of uncomplicated UTIs. In this scenario, it is likely that a number of the clinical isolates from community-acquired UTIs originated from patients whose initial empiric therapy had failed. The presence of such a selection bias represents a general problem in studies addressing the epidemiology of community-acquired uncomplicated UTIs,[33,34] and is even more complicated in resource-limited countries for issues related to weaknesses of healthcare systems and poverty.

3.3. Characterization of the enterobacterial isolates resistant to expanded-spectrum cephalosporins

All 24 isolates that were resistant to expanded-spectrum cephalosporins exhibited a multidrug resistance phenotype (defined as resistance to at least three different classes of antibiotic agents) (Table 3). In particular, susceptibility rates of expanded-spectrum cephalosporin-resistant *E. coli* ($n = 18$) and *K. pneumoniae* ($n = 6$) were as follows, respectively: meropenem, 100% and 100%; fosfomycin, 83% and 83%; nitrofurantoin, 83% and 0%; amikacin, 72% and 67%; trimethoprim–sulphamethoxazole, 33% and 17%; gentamicin, 22% and 0%; tetracycline, 22% and 17%; ciprofloxacin, 6% and 0%; nalidixic acid, 6% and 0% (Table 3).

All ESBL producers were found to harbour genes encoding CTX-M-type enzymes. CTX-M-1 group variants were the most prevalent (15 isolates, 68%), followed by CTX-M-9 group (seven isolates, 32%) and CTX-M-2 group (one isolate, 5%), with one isolate harbouring variants from two groups (Table 3). Sequencing of $bla_{\text{CTX-M}}$ genes identified $bla_{\text{CTX-M-15}}$ and $bla_{\text{CTX-M-65}}$ as the most prevalent variants (Table 3).

The 16 CTX-M-producing *E. coli* isolates belonged to each of the four major phylogenetic groups (A, 38%; D, 31%; B2, 25%; B1, 6%), with no specific association observed between CTX-M allelic variants and phylogenetic groups (Table 3). Of note, all four CTX-M-15-producing *E. coli* isolates belonging to phylogenetic group B2 were assigned to the pandemic clone ST131, with two isolates of hospital origin belonging to the H30-Rx subclone.[28–30,35]

The two isolates producing AmpC-like enzymes were shown to harbour $bla_{\text{CMY-2}}$.

Altogether, the molecular epidemiology of CTX-M enzymes in clinical isolates from the Bolivian Chaco appeared to be coherent with their recent evolution in South America.[1–3,14] The heterogeneity in terms of CTX-M allelic variants and population structure (i.e., diverse *E. coli* phylogenetic groups) would exclude a major role of clonal expansion in the dissemination of CTX-M ESBLs among enterobacteria responsible for UTIs in the study setting, and the investigation of plasmids encoding the most prevalent variant (i.e., CTX-M-15) deserves further attention. Interestingly, the urinary *E. coli* isolates analyzed in this study exhibited a comparable prevalence of CTX-M producers (9% vs. 17%; $p = 0.07$) and a similar distribution of CTX-M variants (CTX-M-15: 56% vs. 38%, $p = 0.4$; CTX-M-65: 19% vs. 27%, $p = 0.8$) as those of commensal *E. coli* collected in 2011 from healthy children living in the same urban area,[8] emphasizing the role of commensal enterobacteria as a reservoir of clinically relevant resistance determinants.

3.4. Detection of emerging aminoglycoside and fosfomycin resistance genes

Acquired 16S rRNA methyltransferases (accounting for high-level and broad-spectrum aminoglycoside resistance) and glutathione S-transferases (accounting for fosfomycin resistance) have been reported increasingly among enterobacterial isolates in recent years, often in association with beta-lactamases, further complicating the management of infections caused by multidrug-resistant isolates.[25,36–39] Although resistance to amikacin and fosfomycin were uncommon in this setting, the isolates non-susceptible to these drugs were screened for the presence of the above resistance determinants.

All isolates exhibiting amikacin non-susceptibility (*E. coli*, $n = 8$; *K. pneumoniae*, $n = 2$) were subjected to a multiplex PCR for the detection of the 10 known 16S rRNA methyltransferase determinants.[25] Positive results for *rmtB* were obtained with the two *K. pneumoniae* isolates. Both isolates also carried $bla_{\text{CTX-M-65}}$, suggesting a possible genetic linkage between the two resistance determinants (not further investigated) (Table 3). The epidemiology of plasmid-encoded 16S rRNA methyltransferases in South America has been poorly investigated so far, with the few data available pointing towards a dominance of RmtD-like enzymes and the occasional occurrence of RmtB (in *E. coli* and *Proteus mirabilis*

Table 3
Phenotypic and molecular features of expanded-spectrum cephalosporin-resistant isolates from the Bolivian Chaco

Isolate	Year	Origin[a]	Other resistance traits[b]	CTX-M group	CTX-M variant[c]	Phylogenetic group[d]	Other relevant resistance genes
E. coli VM-72	2011	H	NAL, CIP, CN, TET	1	CTX-M-15	B2 (H30-Rx ST131)	-
E. coli VM-292	2011	C	NAL, CIP, CN, FOT, TET	9	CTX-M-65	D	*fosA3*
E. coli VM-77	2012	C	SXT, NAL, CIP, CN, TET	9	CTX-M-14	D	-
E. coli VM-82	2012	C	sxt, NAL, CIP, CN, TET	9	CTX-M-14	D	-
E. coli VM-255	2012	C	NAL, CIP, CN	-	-	A	bla_{CMY-2}
E. coli VM-334	2012	H	SXT, NAL, CIP, CN, NIT, TET	9	CTX-M-65	A	-
E. coli VM-337	2012	C	SXT, NAL, CIP, CN, NIT, TET	1	CTX-M-15	A	-
E. coli VM-353	2013	H	FOT, TET	1	CTX-M-55	A	*fosA3*
E. coli VM-363	2013	C	SXT, NAL, CIP, CN, AK, TET	-	-	B1	bla_{CMY-2}, *aac(6′)Ib*
E. coli VM-364	2013	C	SXT, NAL, CIP, CN, ak, TET	1	CTX-M-15	B2 (non-H30 ST131)	*aac(6′)Ib-cr*
E. coli VM-365	2013	C	NAL, CIP, ak	1	CTX-M-15	A	*aac(6′)Ib-cr*
E. coli VM-366	2013	H	SXT, NAL, CIP, CN, TET	9	CTX-M-65	A	-
E. coli VM-379	2013	H	SXT, NAL, CIP, CN, nit, TET	1	CTX-M-15	A	-
E. coli VM-439	2013	H	SXT, NAL, CIP	1	CTX-M-15	B1	-
E. coli VM-444	2013	C	SXT, NAL, CIP, CN, AK, TET	1	CTX-M-15	B2 (non-H30 ST131)	*aac(6′)Ib-cr*
E. coli VM-474	2013	C	SXT, NAL, CIP, CN	1	CTX-M-15	D	-
E. coli VM-517	2013	U	SXT, NAL, CIP, CN, FOT, TET	1	CTX-M-55	D	*fosA3*
E. coli VM-498	2014	H	NAL, CIP, ak, TET	1	CTX-M-15	B2 (H30-Rx ST131)	*aac(6′)Ib-cr*
K. pneumoniae VM-249	2012	C	SXT, NAL, CIP, CN, NIT, TET	1	CTX-M-15	ND	-
K. pneumoniae VM-354	2013	H	SXT, NAL, CIP, CN, NIT, TET	1, 2	CTX-M-2, CTX-M-15	ND	-
K. pneumoniae VM-397	2013	C	NAL, CIP, CN, AK, NIT, FOT, TET	9	CTX-M-65	ND	*fosA3*, *rmtB*
K. pneumoniae VM-419	2013	H	SXT, NAL, CIP, CN, NIT, TET	1	CTX-M-15	ND	-
K. pneumoniae VM-466	2013	C	SXT, NAL, CIP, CN, NIT	1	CTX-M-15	ND	-
K. pneumoniae VM-477	2013	H	SXT, NAL, CIP, CN, AK, NIT, TET	9	CTX-M-65	ND	*rmtB*, *aac(6′)Ib-cr*

[a] H, hospital; C, community; U, unknown.
[b] SXT, trimethoprim–sulphamethoxazole; NAL, nalidixic acid; CIP, ciprofloxacin; CN, gentamicin; AK, amikacin; NIT, nitrofurantoin; FOT, fosfomycin; TET, tetracycline. For the antibiotics, upper case indicates a resistance phenotype and lower case indicates an intermediate phenotype.
[c] All isolates ($n = 24$) were classified as potential extended-spectrum beta-lactamase (ESBL) producers (as per the Clinical and Laboratory Standards Institute (CLSI)) on the basis of inhibition zone diameters of cefotaxime and/or ceftazidime smaller than the screening breakpoints ($\leq$27 mm for cefotaxime and $\leq$22 mm for cefotaxime). The phenotypic confirmatory test for ESBL production (as per CLSI) was positive for 22 isolates using both cefotaxime and ceftazidime, with the exception of the two *E. coli* isolates producing CTX-M-14 (*E. coli* VM-77 and *E. coli* VM-82) for which a $\geq$5 mm increase in inhibition zone diameter in the presence of clavulanic acid was observed only with cefotaxime. The two CMY-2-producing *E. coli* isolates (*E. coli* VM-255 and *E. coli* VM-363) were negative by the modified CLSI confirmatory test for ESBL detection in AmpC producers, as recently proposed by Poulou et al.[21]
[d] ND, not determined.

isolates).[1,40] The present data provide further evidence of the dissemination of RmtB among enterobacterial pathogens from this epidemiological setting.

The amikacin non-susceptible phenotype of the remaining isolates was found to be mostly the result of the presence of *aac(6′)Ib* and its variant *aac(6′)Ib-cr* (detected in one and six isolates, respectively; all but one resistant to expanded-spectrum cephalosporins) (Table 3). These results confirmed the dissemination of *aac(6′)Ib-cr* (clinically relevant for its ability to reduce susceptibility to both aminoglycosides and quinolones) in the study setting,[41] and its frequent association with ESBL determinants.

Among the seven isolates showing a fosfomycin non-susceptible phenotype (*E. coli*, $n = 3$; *Morganella morganii*, $n = 3$; *K. pneumoniae*, $n = 1$), four were identified as producers of glutathione S-transferases by a recently developed disk potentiation test (i.e., all *E. coli* and *K. pneumoniae* isolates),[22] and all of them were found to harbour *fosA3*. Of note, all the *fosA3*-positive isolates were also CTX-M producers (Table 3), confirming the frequent association of *fosA3* with CTX-M determinants.[42–44] The fosfomycin resistance phenotype shown by the three *M. morganii* isolates was not unexpected and confirmed previous reports of a very low susceptibility to this drug.[45]

3.5. Conclusions

In general, antimicrobial chemotherapy for uncomplicated UTIs is selected empirically, based on guidelines that consider the local epidemiology of antimicrobial resistance, which can be very different in diverse geographical settings.[3,33] This is the first study on the antimicrobial susceptibility of clinical isolates from UTIs of patients in the Bolivian Chaco region, and one of the first from Bolivia. The present data indicate an overall high resistance burden, with extremely high levels of resistance to trimethoprim–sulphamethoxazole and fluoroquinolones, and a sizeable presence of ESBL determinants in *Enterobacteriaceae*, suggesting that nitrofurantoin and fosfomycin might represent the first-line treatment options for uncomplicated UTIs in this area.[3,33]

This is also the first study to characterize antibiotic resistance determinants in enterobacteria responsible for UTIs in Bolivia. Among relevant findings, the emergence of FosA3 among ESBL-producing isolates was noted and is of concern, since it could impair the efficacy of fosfomycin. The other alarming finding was the emergence of RmtB, which compromises the activity of all aminoglycosides, which in a similar setting could be important options for complicated UTIs caused by multidrug-resistant pathogens.

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Conflict of interest: The authors declare that they have no competing interests in relation to the results presented in this study.

Authors' contributions: AB and GMR conceived and coordinated the study, analyzed the results, and wrote the manuscript. TDM and AM carried out bacterial identification and susceptibility testing. SS, ER, and LP carried out phenotypic and molecular detection and characterization of resistance determinants, and LP also drafted the manuscript. ALV provided the clinical isolates and helped to draft the manuscript. MS participated in the design of the study, coordinated the field activities, and performed the statistical analysis. CR participated in data analysis and critically revised the manuscript. All authors read and approved the final manuscript.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ijid.2015.12.008.

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