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AmpC, oprD Expression Analysis in β-lactam Resistant *Pseudomonas aeruginosa* Clinical Isolates from a Tertiary Level Hospital in Ecuador

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ABSTRACT.- Innate and acquired antibiotic resistance mechanisms in *Pseudomonas* present a challenge for clinicians looking for timely and effective chemotherapy. This is particularly important in critical care hospital settings. This study is aimed at achieving a deeper understanding of two of the most important drug resistance mechanisms in *Pseudomonas aeruginosa* at the molecular level. One hundred clinical isolates of *Pseudomonas aeruginosa* were obtained from a tertiary level hospital in Quito, Ecuador. Expression of *ampC* and *oprD* was analysed through quantitative real-time PCR assays. A comparison between the *ampC* and *oprD* expression profiles and the phenotypes in antimicrobial susceptibility testing (AST) was conducted, with more than 50% of the isolates having concordant profiles for both *ampC* and *oprD* expression. Our results suggest that *ampC* and *opr*D expression might provide useful information about molecular resistance mechanisms in strains which are circulating in Ecuador. However, larger scale studies could clarify drug resistance mechanisms in order to guide targeted treatment.

KEYWORDS: *ampC, oprD*, β-lactams, *Pseudomonas aeruginosa*, RT-qPCR.

RESUMEN.- Los mecanismos innatos y adquiridos de resistencia a los antibióticos en *Pseudomonas* representan un reto para los médicos que buscan una quimioterapia oportuna y eficaz. Esto es particularmente importante en las áreas de cuidados intesnsivos de los hospitales. Este estudio está dirigido a lograr una comprensión a nivel molecular de dos de los más importantes mecanismos de resistencia a los fármacos en *Pseudomonas aeruginosa*. Cien aislados clínicos de *Pseudomonas aeruginosa* se obtuvieron de un hospital de tercer nivel en Quito, Ecuador. Se analizó la expresión de *amp*C y *opr*D mediante PCR cuantitativa en tiempo real. Se realizó una comparación entre los perfiles de expresión *amp*C y *opr*D y los fenotipos obtenidos en la prueba de susceptibilidad antimicrobiana (AST), con más del 50% de los aislados con perfiles concordantes para la expresión *amp*C y *opr*D. Nuestros resultados sugieren que la expresión *amp*C y *opr*D podría proporcionar información útil sobre mecanismos de resistencia molecular en cepas que están circulando en Ecuador. Sin embargo, los estudios a mayor escala pueden aclarar los mecanismos de resistencia a los fármacos para establecer el tratamiento adecuado.

PALABRAS CLAVES: *ampC*, β-lactams, *oprD*, *Pseudomonas aeruginosa*, RT-qPCR.

INTRODUCTION

Pseudomonas aeruginosa is one of the most commonly isolated Gram negative bacteria in hospital-acquired infections. Multi-drug resistant strains are a growing problem in health care settings, especially in extremely ill patients at intensive care units (Obritsch et al. 2004). Eradication of the infection is complex due to the intrinsic capacity of *P. aeruginosa* to decrease its membrane permeability, to express efflux pumps, to produce antimicrobial-degrading enzymes and to acquire new resistance mechanisms through mutations or plasmids (Luján Roca 2014).

The genome of the wild type *P. aeruginosa* strain (PAO1) revealed the presence of an unusually high number of genes involved in the regulation of bacterial pathogenicity (Kipnis et al. 2006 Stover et al. 2000 Woods 2004). For instance, the OprD channel allows access to carpabenems such as meropenem and imipenem (Hancock and Brinkman 2002, Nikaido 1989). OprD-mediated resistance might be related to different mechanisms that decrease expression of a normally functioning porin, including mutations that cause translational disruptions (Yoneyama and Nakae 1993). Furthermore, the production of AmpC β-lactamase confers resistance to most penicillin, cephalosporin and cefamicins and shows variable results for aztreonam. However, the expression at basal levels of *ampC* is not enough alone to determine anti-pseudomonal β-lactams resistance (Giwercman et al. 1990) Overexpression of *ampC* and regulation of the factor AmpR are necessary to acquire a resistant phenotype (Jacoby 2009).

A five-year report (1997–2001) of the SENTRY Antimicrobial Surveillance in the Latin American region found a significant rise of resistance rates to commonly used anti-pseudomonal antibiotics over a relatively short period of time (Andrade et al. 2003). In 2008 in Ecuador, the National Antimicrobial Resistance Network (REDNARBEC) reported antibiotic resistance rates ranging from 25% for amikacine to 45% for aztreonam, with even higher rates in intensive care units. However, the majority of the available local studies on antimicrobial resistance are based on phenotypic profiles (AST). For this reason, we wanted to achieve a better understanding of the molecular mechanisms involved in antimicrobial resistance in Pseudomonads in clinical settings. The methodology used for this purpose is described in the next section of this study.

The aims of this study were thus (i) to analyze *ampC* and *oprD* expression levels, and (ii) to compare gene expression profiles for susceptible and antibiotic-resistant *Pseudomonas aeruginosa* isolates obtained from a referral hospital in Ecuador in order to clarify their molecular mechanisms of resistance.

MATERIALS AND METHODS

Clinical isolates and controls.- One hundred *P. aeruginosa* clinical isolates were collected from the Bacteriology Laboratory, Carlos Andrade Marin Hospital (HCAM) in Quito, one of Ecuador's largest hospitals. Due to their genotypic profiles, the clinical isolates P.a.119 is an antibiotic susceptible and P.a.5 is an antibiotic resistant control. Additionally, six of the isolates were unable to grow. Consequently, this study was performed using 92 clinical isolates that were recovered either from their corresponding culture plate from AST plates, which included 5 anti-pseudomonal agents (ATM: aztreonam; CAZ: ceftazidime; TPZ: piperacilin-tazobactam; IMP: imipenem and MEM: meropenem) or from the primary clinical isolate in MacConkey agar, depending on isolate availability in the hospital lab. AST was performed in the Bacteriology Lab by qualified microbiology technicians. Susceptibility break points followed the accepted lab standards at the time (CLSI). Results from the full AST panels are not included in this study. Upon collection, a single colony from each clinical isolate was transferred to BD Pseudomonas Isolation Agar (DIFCO, Sparks). Bacterial suspensions (in glycerol 15%) were then deep frozen at -80°C.

Primer and probe design.- Four sequence alignment runs were performed with the Clustal X 2.0 software (European Bioinformatics Institute, 2007) for *ampC*, *oprD* and *rpsL* (used as normalizer in this study) gene sequences of the *P. aeruginosa* reference strains PAO1 (ID:878149, ID:881970, ID:881701), PA7 (ID:5353514; ID:5358647; ID: 5356699, UCBPP (ID:4382097;ID:4380780; ID:4381343), and LESB58 (ID:7175766; ID: 7180952). Sequences were obtained from the GeneBank database (National Centre for Biotechnology Information) and the Pseudomonas Genome Database (Winsor et al. 2011). A combination of primers and Taqman® probes were designed by using the Primer Express® software package (Applied Biosystems, USA), Olygo Analyzer (Integrated DNA Technologies) and BLASTn (National Library of Medicine). (See Table S1 Supporting information.)

RNA isolation.- 100μL of the stored bacterial sus-

pensions was added to 5mL of Brain Heart Infusion Broth Media (DIFCO, Sparks) followed by incubation at 37°C with continuous shaking (200rpm) until an optical density (OD600) of 0.8-1 was reached. RNA isolation from cultures was then carried out with the PureLink Micro-to-Midi Total RNA Purification System (Life Technologies, California) according to manufacturer instructions, with a few modifications. Samples were treated with RNA Stabilization Reagent (Qiagen) before RNA isolation with a centrifugation step at 4°C. DNase treatment was then performed (DNase I, Ambion, Austin, USA) followed by a final enzyme deactivation step with EDTA 5mM and a purification step with 2M LiCl. RNA quality and yield were assessed by spectrophotometry (NanoVue®, General Electric, Cambridge, MA).

RT-qPCR.- RT-qPCR mixture (10µL) consisted of 100ng of RNA template. For rpsL final concentrations were 300nM for the forward primer and 50nM for the reverse primer. Final probe concentration in every system was 2000nM, (Applied Biosystems, USA). Reaction mixture included 5µL of 2X Taqman mix from RNA-to-CT One Step TM One Step Kit (Applied Biosystems, USA), 0.125µL of 40X Taqman® RT Enzyme Mix (Applied Biosystems, USA), and nuclease free water to complete the final volume. RT-qPCR was performed in a 7300 Real Time PCR System (Applied Biosystems, USA), with initial retro transcription at 48°C for 15 minutes, enzymatic activation at 95°C for 10 minutes, followed by 40 cycles that consisted of a denaturation step at 95°C for 15 seconds and an annealing-extension step at 60°C for 60 seconds.

Expression analysis.- The adjusted *ΔΔCt* method proposed by Yuan et al. (2006), was used in this study. An effect on gene transcription was considered significant when expression levels were ≥2.5 and ≤0.4-fold. A threshold level of 20-fold relative to the *ampC* expression level in susceptible strain P.a.119 allowed for discrimination of susceptible and resistant strains. The expression level was calculated relative to P.a.5 expression. Since the *oprD* assay evaluated presence or absence of the porin, for this assay, the threshold was set at 1-fold. ΔCt values for susceptible (P.a.119) and resistant (P.a.5) controls using four different concentrations (10; 8; 0.4 and 0.08ng/µL) were tested for the F variance test with a p value of >0,05. ΔCt median values (see Table S2 Supporting Information) tested with t and Wilcoxon tests showed a difference between susceptible and resistant isolates used as controls with p values of < 0.05. Expression levels were then calculated by using the ΔΔCt adjusted method and $2^{-\Delta\Delta Ct}$adjusted (Yuan et al. 2006). Qualitative analysis and genotype assignment for susceptible and resistant isolates were based on the above cut-offs (20-fold for ampC and 1-fold for oprD). For expression levels of the (0<X<1)-fold genotype assignment was based on the phenotypic AST profile (see Table S3 Supporting Information).

Statistical analysis.- The means of ΔCt from sensitive and resistant strains were compared using t and Wilcoxon tests. Statistical analyses were performed using the InfoStat package (Universidad Nacional de Córdoba, Argentina), Microsoft Excel (Microsoft Corp.) and Origin 8.0 (OriginLab).

RESULTS

AST Profiles.- Inhibition diameters were used for definition of antibiotic susceptibility in compliance with National Committee for Clinical Laboratory Standards breakpoint recommendations. The results in Figure 1 show that more than 50% of the total isolates were susceptible to four of the drugs. In contrast, the percentages for the aztreonam (ATM) susceptible and resistant isolates were 43.5% and 44.6%, respectively.

Gene expression analysis.- Phenotype/genotype correlation tests revealed discordance among antibiotic susceptibility profiles. Isolates with a susceptible/resistant genotypic profile matching their appropriate AST profile were labelled as "concordant". Results on Figure 1 show average concordance values (%) for each antibiotic. Qualitative evaluation of susceptibility tests and *ampC* expression levels showed an average of 55.9% (Figure 1A) concordant strains, while for *oprD* this figure was 50.1% (Figure 1B). For *ampC*, the average expression level was 5.6-fold for susceptible isolates and 1234.42-fold for resistant isolates.

DISCUSSION

We measured *oprD* and *ampC* expression levels with an qRT-PCR assay and compared the expression profiles obtained to the isolates' AST profiles. As expected, this study showed a discordant phenotype/genotype profile in 53 % of the isolates. Similar results have been found in Ecuador (unpublished data) where it was shown that 45% of clinical strains of *P. aeruginosa* were discordant when comparing AST and molecular testing.

Chromosomal expression of AmpC *β*-lactamase

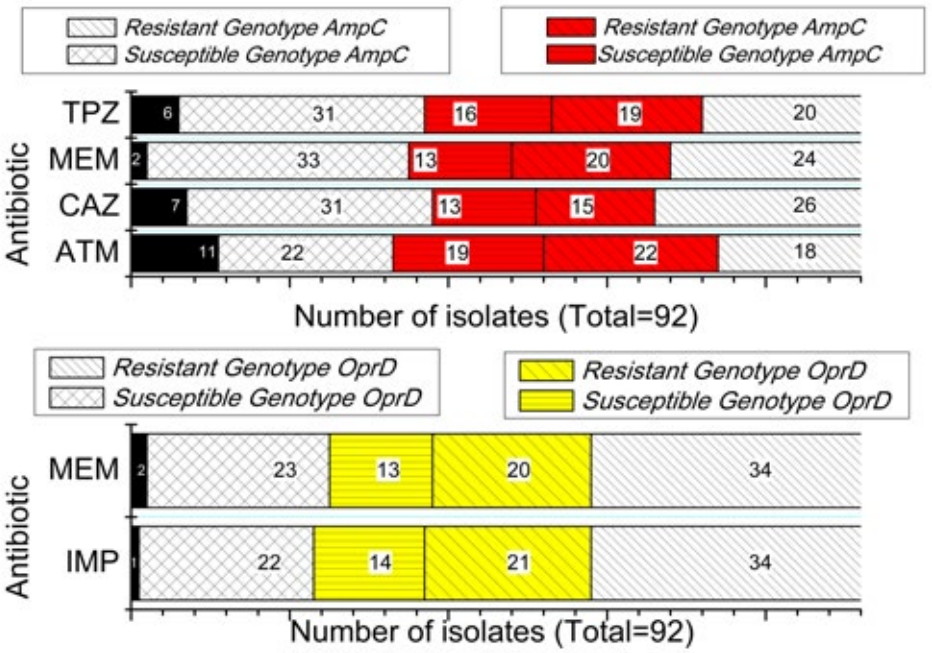

Figure 1. Antibiotic Susceptibility Testing (AST) phenotype/genotype analysis for *AmpC* and *OprD*. Phenotype (AST): Red/Yellow-Resistant, White-Susceptible, Black-Not available. A) AST profile result (phenotype) and correlation with *AmpC* expression levels (genotype) for: Piperacilin-Tazobactam (TPZ), MEM (meropenem), Ceftazidime (CAZ) and Aztreonam (ATM) in 92 clinical isolates of *P. aeruginosa*. Concordance averages for the above antibiotics are 57.3%, 58.6%, 53.5% and 54.3%, respectively. B) AST profile result (phenotype) and correlation with *OprD* expression levels (genotype) for: Imipenem (IMP) and Meropenem (MEM) in 92 clinical isolates of *P. aeruginosa.* Concordance averages for the above antibiotics are: 49.6% and 50.5%, respectively.

confers resistance to penicillin and cephalosporin in *P. aeruginosa.* In this study, resistant strains for CAZ (36.6%) and TPZ (48.7%) might be overexpressing AmpC. This resistance mechanism has been previously described as a complete or partial de-repression of this gene (LucDumas et al. 2006).

Despite the fact that aztreonam resistance in 53.7% of the isolates may be mediated by a hyper-production of AmpC, it is important to note that ATZ resistance in *P. aeruginosa* involves other mechanisms, such as the up regulation of efflux pumps (MexAB-OprM, MexCD-OprJ, MexEF-OprN and MexXY-OprM) (Livermore 2002). An explanation based solely on the study of the expression of one gene will therefore not cover all the possibilities of resistance mechanisms in *P. aeruginosa.*

Diffusion of carbapenems (MEM/IMP) inside cells is mediated by the OprD porin. Resistant mutants have reduced expression levels of this porin, or have even led to a complete absence of the channel (LucDumas et al. 2006). For instance, imipenem resistance (38.2%) may have inactivating mutations in the OprD channel that would reduce/block drug intake. Similarly, in inmeropemem resistance for *ampC* (45.5%) and *oprD* (37%), the presence of two different mutations may increase the possibilities of a multi-resistant strain.

Twelve clinical isolates were resistant to both carbapenems, probably due to a synergistic effect between the overexpression of AmpC and an MexAB-OprM efflux pump (Cabot et al. 2011). This strong connection of the different molecular mechanisms demonstrates the complex nature of *P. aeruginosa.*

It is important to note that 47% of the isolates have discordant phenotype-genotype profiles. This discordance can be explained on the basis of transcriptional events such as disruptions of the *oprD* promoter, premature termination of *oprD* transcription or decreased transcriptional expression. Furthermore, carbapenem resistance cannot be specifically linked to AmpC overproduction, due to the complex interplay between resistance mechanisms and the high number of co-regulatory pathways, especially in clinical isolates with a wider genetic and environmental background (Yoneyama and Nakae 1993)

Those isolates which had a "susceptible" phenotype, but that were labelled resistant by molecular analysis, could become resistant months after the initial analysis. *P. aeruginosa* might develop resistance during therapy, even with careful β-lactam drug selection based on initial susceptibility data. This inducibility feature in resistant *P. aeruginosa* has been observed in patients treated with antipseudomonal penicillin, penicillin-inhibitor combinations, AZT and extended-spectrum cephalosporin (Lister et al. 2009). In those isolates that showed genotypic resistance while being phenotypically susceptible, the role of the MexAB-OprM efflux pump, or AmpC overproduction due to an inducer such as IMP (Lister et al. 2009) could be of importance. High expression of *mexA* leavds to resistance to AZT, TPZ and MEM (Lee and Ko 2012). Ceftadizime resistant isolates, however, might have a high expression of *mexB* and *mexY* (Cabot et al. 2011). *P. aeruginosa* strains with a high level cephalosporinase phenotype show higher levels of resistance to CAZ than AZT, which could be also found in strains showing a metallo-β-lactamase phenotype. Strains with the MexA–MexB–OprM active efflux phenotype had a higher level of AZT resistance than CAZ resistance, which is similar to the pattern observed in clinical isolates with the high-level penicillinase phenotype (Vedel 2005).

P. aeruginosa possesses several innate and acquired antibiotic resistance mechanisms (Shen et al. 2015). In this study we analyzed the expression of *ampC* and *oprD* genes in phenotypically susceptible and resistant β-lactam clinical isolates. We then assigned an S/R genotype based on gene expression levels and compared the assigned genotypes with the AST phenotypes. A high level of discordance was found for phenotype/genotype. The presence of other mechanisms such as efflux pumps, plasmids or gene up/down regulation would explain the discordance. Genetic information from this study might be useful for understanding clinical outcomes on the basis of the inducibility of resistance in genetically "resistant" and phenotypically susceptible isolates. Data from this study could form the basis for the rational use of more complex and comprehensive platforms for the study of antibiotic resistance mechanisms at the hospital level.

CONCLUSIONS

This study tested the value of using a limited number of molecular resistance assays for the rapid discrimination of susceptible and resistant *Pseudomonas aeruginosa* clinical strains. The study found that β-lactam resistance patterns, among tested clinical isolates, cannot be fully explained by AmpC overexpression and the low expression of *oprD*. In strains exhibiting phenotypic resistance and genotypic susceptibility, other mechanisms or interactions might be involved. Molecular biology analyses revealed a genetic resistance background in a number of AST susceptible strains, which may play a role in the outcome of a number of infectious episodes. AST phenotyping will need to be complemented by rapid tests at the hospital level. Information from studies like this one might be useful for a comprehensive understanding of the mechanisms involved in antibiotic resistance in *Pseudomonas aeruginosa,* and for the guidance of infection control strategies and rational antibiotic usage.

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Supplementary information

Table S1. Primers and probes used in this study

	Name	Start	End	Sequence 5'- 3'
Primers	rpsL F	109	129	GTA TAC ACC ACC ACG CCG AAA
	rpsL R	208	189	CAC CAC CGA TGT ACG AGG AA
	ampC F	183	206	AGG AGA ACC GCA TTA CTT CAG CTA
	ampC R	277	260	TGC TCA CCG AGC CGA TCT
	oprD F	1062	1083	CCT GAC TTT CAT GGT CCG CTA T
	oprD R	1161	1142	ATC CTC GCC GTA GCC GTA GT
Taqman Probes	rpsL P	159	184	CCG TGT ACG TCT GAC CAA CGG TTT CG
5'FAM-3'TAMRA	ampC P	215	235	CCT CGA AAG AGG ACG GCC GCC
	oprD P	1098	1124	CAT CGA TGG CAC CAA GAT GTC TGA CAA

Table S2. Calculation of ΔCt, derived from subtracting Ct number of reference gene from that of the target gene using independent samples.

Isolate	Gene	Group	ngDNA/uL	Ct	Isolate	Gene	Group	ngDNA/uL	Ct	ΔCt
P.a.119	*ampC*	1	10	26093	P.a.119	*rpsL*	3	10	191623	7047
			2	29100				2	207571	8343
			0.4	31450				0.4	23371	8079
			0.08	33508				0.08	260866	7364
P.a.5	*ampC*	2	10	21161	P.a.5	*rpsL*	4	10	197510	1409
			2	23456				2	21629	1827
			0.4	26035				0.4	243083	1727
			0.08	282939				0.08	269142	1379

Table S3. Relative expression analysis for all isolates tested. AST analysis is based on CLSI standards. S represents sensitive and R resistant. Genotypic analysis is based on cut off points for ampC =20-fold and for oprD=1-fold (values (0<X<1) required validation based on AST for genotype assigment).

		PHENOTYPIC ANALYSIS					GENOTYPIC ANALYSIS	
No. Isolate	**Lab Sample Code**	**ATM**	**CAZ**	**IMP**	**MEM**	**TPZ**	***ampC***	***oprD***
P.a.2	11021115	S:25	S:24	S:23	S:27	S:23	**S:0.335**	**S:12.36**
P.a.3	11021116	S:26	S:26	S:26	S:24	S:23	**S:0.028**	**S:15.6**
P.a.4	10273249	R:11	S:23	S:24	S:24	S:21	**S:0.087**	S:0.913
P.a.7	11131081	ND	S:23	S:26	S:30	ND	**R:110.445**	**S:38.08**
P.a.8	11141338	R:16	S:23	S:24	S:26	S:22	**S:5.692**	**R:0**
P.a.9	11141248	R:6	R:18	S:27	S:24	R:6	S:1.608	**R:0**
P.a.10	11151194	S:23	S:26	S:28	S:24	S:22	**S:0.016**	S:1.974
P.a.11	11151193	ND	ND	S:23	S:23	S:21	**S:16.90**	**R:0**
P.a.12	11171255	S:23	S:23	S:25	S:26	S:28	S:1.507	**R:0**
P.a.14	11181146	S:22	S:26	S:28	S:32	S:24	**S:2.696**	**R:0**
P.a.15	11251191	R:16	R:14	S:23	S:28	S:22	**S:2.637**	S:2.334
P.a.16	11181252	ND	R:14	S:23	S:26	R:16	**S:2.596**	**R:0**
P.a.18	12071319	R:14	S:23	S:24	S:23	R:10	**S:0.262**	**R:0.880**
P.a.19	12091227	R:6	S:18	S:18	R:6	R:12	**R:150.270**	**R:0**
P.a.20	12081157	R:21	S:23	S:23	S:29	R:16	**S:5.430**	**R:0**
P.a.21	12231243	S:24	S:26	S:24	S:22	S:22	**R:3772.747**	**S:3780.6**
P.a.22	12161191	ND	R:14	R:10	R:10	R:14	**R:6966.656**	**R:0**
P.a.23	12201287	S:32	S:28	S:24	S:24	ND	**S:13.474**	S:1.75
P.a.24	12193049	R:14	ND	S:30	S:25	R:15	**R:108.961**	**R:0.02**
P.a.25	12151218	R:12	S:23	S:25	S:26	S:23	**R:30.116**	**S:6.56**
P.a.26	12140634	ND	S:26	S:23	S:25	S:21	S:2.132	**R:0**
P.a.27	12291217	R:10	S:22	R:10	R:6	R:15	**R:59.506**	**R:0**
P.a.28	1021188	R:6	R:10	S:23	S:24	ND	**R:65.527**	**R:0**
P.a.29	1031219	S:23	S:26	S:24	S:30	S:32	**S:0.173**	**R:0**
P.a.30	1031277	ND	R:6	S:24	S:23	R:14	**R:172.671**	**R:0**
P.a.31	1031304	R:14	S:25	R:15	R:14	R:6	**S:5.207**	R:0.722
P.a.32	12301270	ND	R:12	R:10	R:10	R:13	**R:1766.666**	**R:0**
P.a.34	1100659	R:6	R:6	R:10	R:6	R:6	**R:76.033**	**R:0**
P.a.35	1031363	R:10	S:22	S:24	R:6	R:15	S:2.022	**R:0**
P.a.36	1110461	S:30	S:30	S:30	S:30	S:32	**S:0.268**	**R:0**
P.a.49	1231373	S:24	S:24	S:29	S:29	S:21	**R:33.255**	**R:0**
P.a.51	1201208	ND	S:28	S:26	S:30	S:28	**S:0.302**	**R:0**
P.a.52	1181297	S:23	S:27	S:25	S:30	S:25	**S:11.877**	**R:0**
P.a.53	1238029	R:6	R:6	R:12	R:10	R:11	**R:215.432**	**S:2.55**
P.a.54	1201226	R:13	S:18	R:12	R:6	S:20	**R:718.823**	**S:740.44**
P.a.55	1180460	R:6	S:22	S:30	S:20	S:18	**R:359.920**	**R:0**
P.a.58	1241228	R:12	R:16	R:14	R:11	R:11	**S:12.328**	**S:3.10**
P.a.59	1162013	S:24	S:26	S:30	R:14	ND	R:49.583	S:0.52
P.a.60	1198025	ND	S:24	R:14	R:11	R:11	**S:3.070**	R:2.382
P.a.61	1208034	R:12	S:21	R:14	R:12	R:10	**R:20.207**	**R:0**
P.a.72	1161270	R:6	R:6	R:10	R:15	R:10	**R:160.548**	**S:29.89**
P.a. 73	1161274	R:6	R:10	R:18	R:18	S:24	**S:0,77**	**R: 0.65**
P.a.74	1161262	R:6	R:6	R:10	S:16	R:10	**S:0.011**	**S:35.96**
P.a.75	1241286	R:6	R:6	R:10	R:12	S:28	**S:0.364**	S:1.728
P.a.77	1233168	R:8	S:23	S:24	S:18	S:18	**S:4.516**	**R:0.014**
P.a.78	1301303	S:24	S:25	ND	ND	S:24	**S:11.029**	**R:0.12**
P.a.79	1311208	R:6	S:22	R:12	R:12	S:22	S: 2.078	**R:0**
P.a.80	1201226	R:13	S:18	R:12	R:6	S:20	**R:158.839**	**R: 0.55**
P.a.82	1300431	R:10	S:22	R:10	R:10	S:23	**R:38.935**	**R:0**
P.a.83	2068047	S:24	R:17	R:15	R:6	R:10	**R:288.281**	**R:0.17**
P.a.84	2068036	S:24	R:16	S:17	R:6	R:16	**R:6037.321**	**S:12.18**
P.a.85	2081282	R:11	R:11	R:13	R:6	R:14	**R:390.412**	S: 2.336

Table S3 continuation

P.a.86	2103266	S:28	S:23	S:26	S:28	S:24	**S:9.525**	**R:0**
P.a.87	2031228	S:22	R:15	R:14	R:12	S:21	**S:0.00**	**R:0**
P.a.88	2131285	R:6	R:6	R:6	R:6	R:11	**R:186.993**	**S:22.58**
P.a.89	2111135	R:6	R:6	R:8	R:6	R:11	S:0.716	**R:0**
P.a.90	2061320	S:24	R:16	S:25	S:20	R:17	**S:0.398**	**S:13.47**
P.a.91	2100658	S:25	S:25	S:25	S:30	S:25	**R:86.398**	**S:14.43**
P.a.92	2141215	R:16	R:18	S:28	S:28	S:24	**S:0.343**	S:1.636
P.a.93	2091239	R:16	S:23	S:28	S:28	S:30	**R:193.169**	**R:0**
P.a.94	3051324	R:6	R:6	R:12	R:10	ND	**R:24.729**	**S:40.23**
P.a.95	3051325	R:6	R:6	S:28	S:24	R:10	**S:19.376**	**R:0**
P.a.96	3051319	S:24	S:25	S:25	S:29	S:23	**S:10.943**	**S:4.62**
P.a.97	3063111	ND	S:27	S:35	S:31	S:23	**S:4.075**	S:1.249
P.a.98	3051327	R:12	R:15	S:28	S:24	R:10	**R:114.579**	**R:0**
P.a.99	3060519	R:6	S:24	S:26	ND	S:21	**S:2.561**	**R:0**
P.a.100	3091135	R:15	R:8	R:12	R:6	R:10	**R:684.617**	**S:5.11**
P.a.101	3050286	S:22	S:22	R:12	R:12	R:6	**S:3.818**	**R:0.809**
P.a.102	3138003	R:6	S:24	R:6	R:6	R:6	**R:63.499**	**R:0.708**
P.a.103	3181108	ND	R:6	R:6	R:6	R:6	**S:3.840**	**S:4.24**
P.a.104	3161171	S:24	S:25	R:10	S:25	ND	**R:24.198**	**R:0**
P.a.105	3201336	R:12	S:18	R:12	R:6	R:15	**S:9.046**	**R:0**
P.a.106	3211222	R:18	S:24	R:15	R:10	R:6	**R;552.38**	**S:18.33**
P.a.107	3071352	S:26	S:26	R:12	R:13	R:16	**S:0.149**	**R:0**
P.a.111	4091355	S:27	ND	S:25	S:28	S:23	**S:12.431**	S:1.267
P.a.112	4091345	S:34	S.35	R:12	S:16	S:24	**R:392.668**	**S:8.46**
P.a.113	4091339	S:32	S:30	R:14	S:16	S:24	**S:3.120**	**R:0.024**
P.a.114	4031360	S:28	S:30	R:15	S:20	S:23	**S:14.808**	**R:0.35**
P.a.115	4091347	S:34	S.35	R:12	S:16	S:24	**R:341.736**	**S:9.27**
P.a.116	4091418	S:32	ND	S:30	S:30	S:24	**S:9.248**	**R:0**
P.a.117	4161402	S:26	ND	S:30	S:28	S:28	**R:250.292**	**R:0**
P.a.118	4121276	S:25	S:30	S:25	S:28	S:24	**R:23.588**	**R:0**
P.a.120	4111697	S:30	S:25	S:28	S:30	S:24	**R:900.241**	**S:221.31**
P.a.121	3271265	S:25	S:28	S:24	S:30	S:22	**R:226.113**	**R:0**
P.a.122	4231360	R:20	R:6	S:23	S:25	R:12	**R:18995.028**	**R:0**
P.a.123	4171326	S:28	ND	S:25	S:25	S:25	**R:7062.392**	**S:224.86**
P.a.124	4241507	S.28	S:27	S:28	S:28	S:28	**R:170.923**	**R:0.33**
P.a.125	4181242	S:25	S:25	S:28	S:30	S:25	**S:8.658**	**S:3.63**
P.a.126	4171314	S:22	S:23	S:25	S:28	S:23	**R:116.421**	**S:3.28**
P.a.127	4181238	S:25	S:25	S:38	S:30	S:25	**R:205.875**	**S:33.20**
P.a.128	4191192	S.28	ND	S:25	S:25	S:25	**S:0.00**	**R:0**
P.a.129	4251282	S:24	S:28	S:28	S:28	S:22	**R:80.724**	**R:0**