UNIVERSIDAD SAN FRANCISCO DE QUITO

Colegio de Postgrados

Evidence of Horizontal Gene Transfer of Antibiotic Resistance Genes in Communities with Limited Access to Antibiotics
(el idioma de esta tesis es inglés)

Rosana Segovia

Tesis de grado presentada como requisito para la obtención del título de Magíster en Microbiología

Quito, marzo 2008

UNIVERSIDAD SAN FRANCISCO DE QUITO

Colegio de Postgrados

HOJA DE APROBACION DE TESIS

Evidence of Horizontal Gene Transfer of Antibiotic Resistance Genes in Communities with Limited Access to Antibiotics

(*el idioma de esta tesis es inglés*)

Rosana Segovia

Gabriel Trueba, Ph.D.
Director de la Maestría en Microbiología y
Director de Tesis

....

Venancio Arahana, Ph.D.
Miembro del Comité del Tesis

....

Marco Fornassini, M.D. Ph.D-
Miembro del Comité del Tesis

....

Stella de la Torre, Ph.D.
Decano del Colegio de Ciencias Biológicas y Ambientales

....

Víctor Viteri Breedy, Ph.D.
Decano del Colegio de Postgrados

....

Quito, marzo 2008

DEDICATORIA

A mis padres, Maggi y Jorge Segovia, a mi esposo Diego Granja.

AGRADECIMIENTOS

Quisiera agradecer a Lee Riley por proveer la cepa *E. coli* K12 y por su asesoría técnica, a Owen Solberg por su ayudar con el software de GelCompar, a María Elena Peñaranda, Marcia Triunfol, y Robert Beatty por sus consejos en la edición del documento, a Gabriel Trueba por su guía en estos siete años y medio de estudio, a Venancio Arahana y Marco Fornassini por aceptar ser miembros del Comité de Tesis y su ayuda en la corrección del documento, a Verónica Barragán por su asesoría técnica, a Patricio Rojas y Paúl Cárdenas por su contribución en el trabajo del laboratorio, a Dimitri Kakabadse por ser mi asistente en este estudio, y a Daysi Parrales por su ayuda incondicional con el material de laboratorio. Un agradecimiento adicional al apoyo incondicional de mis padres.

Index

RESUMEN

Un problema de salud pública constituye el aumento de resistencia a antibióticos en las bacterias que se atribuye al uso excesivo de antibióticos. Los genes que se encargan de conferir esta resistencia se diseminan mediante expansion clonal o mediante transferencia horizontal de genes (HGT). Sin embargo, estos genes también han sido encontrados en comunidades de Bolivia que tienen un acceso limitado a antibióticos. En este estudio, se investigó la presencia y diseminación de genes de resistencia a antibióticos en 250 aislados de *Escherichia coli* provenientes de muestras fecales obtenidas de dos comunidades remotas en Ecuador. La expansión clonal se probó por campo pulsado (PFGE), y la transferencia horizontal de genes se evaluó por el análisis de RFLPs de integrones y por el secuenciamiento de genes de beta-lactamasas. A pesar del limitado acceso a antibióticos, 32% de los aislados colectados fueron resistentes a al menos uno de los antibióticos probados. De estos aislados, se logró detectar transferencia horizontal de genes en 11% de los aislados (9 de 80). Estos resultados sugieren que la transferencia horizontal de genes es un proceso importante en la diseminación de genes de resistencia a antibióticos en *E. coli* y que la presencia de cepas resistentes de *E. coli* no se restringe a ambientes con excesivo uso de antibióticos.

ABSTRACT

Antibiotic resistance genes are disseminated by clonal expansion or by horizontal gene transfer (HGT), creating a major public health issue that is attributed to excessive use of antibiotics. However, these resistance genes have also been found in communities in Bolivia that have limited access to antibiotics. In the present study, we investigated the presence and dissemination of antibiotic resistance genes in 250 samples of *Escherichia coli* isolates collected from fecal samples in two remote communities in Ecuador. Clonal expansion was tested by PFGE, and HGT was assessed by both integron RFLPs analysis and by β-lactamase genes sequencing. Despite limited access to antibiotics, 32% of the samples collected in the two remote communities were resistant to at least one antibiotic tested. Of these, we were able to detect evidence of HGT in 11% of the samples (9 of 80). These results suggest that HGT may play an important role in disseminating antibiotic resistance genes in *E. coli* and that occurrence of antibiotic resistant *E. coli* is not restricted to environments where antibiotics are used in excess.

1. INTRODUCTION

Increasing bacterial antibiotic resistance related to extensive use of antibiotics in humans and animals constitutes a growing public health concern (1). Extensive antibiotic use has led to a positive selection of cells that carry efficient mechanisms of drug resistance. Such mechanisms include the presence of altered target molecules or genes that modify, destroy and remove antibiotics from the cell cytoplasm (20).

1.1. Mechanisms of antibiotic resistance spread in a bacteria population.

The growing presence of multi-drug resistant phenotypes in Enterobacteriaceae that are associated with clinical cases of urinary tract infection, pneumonia, meningitis, and bacteremia have been well established (5-6, 13, 18). In addition, it is known that this increase occurs primarily through clonal expansion or transfer of antibiotic resistant genes from one bacterium to another, a process of gene transfer known as horizontal gene transfer (HGT). The importance of HGT in the spread of antibiotic resistance has been inferred by studies done in bacteria located in different geographic regions (3). These studies showed that bacteria in different human populations carry different antibiotic resistance alleles. However, no direct evidence of HGT in a population has been demonstrated.

The lumen of the intestines with abundant bacterial species provides ideal conditions for the evolution and spread of antibiotic resistance genes by HGT (1, 33).

Under simulated intestinal conditions, the transfer of genes among enterobacteria by HGT has been shown to occur primarily through the transfer of plasmids (14, 4**).**

The ability of bacteria to engage in horizontal gene transfer may be inferred by analyzing the genomic make up of different bacteria. It has been reported that the genome of *E. coli* contains 24.98% horizontally transferred genes (31) whereas in *Bacteroides fragilis* horizontally transferred genes account for only 5.5% of the genome (33). Consequently, *E. coli*, despite being a minor component of the intestinal microbiota (< 0.1%) (9), may play an important role in the dissemination of antibiotic resistance genes.

1.2. Genetic structures that contain resistance genes.

To be protected from environmental threats such as toxic compounds, bacteria can acquire phenotypic protection by transferring genetic elements, including antibiotic resistance genes, among themselves (15, 23). Many antibiotic resistance genes in *E. coli* and in other members of the Enterobacteriaceae family are found on conjugal plasmids as integrons. Integrons are genetic structures with a complex arrangement of gene cassettes that are capable of capturing additional gene cassettes. These gene cassettes are promoterless mobile elements made up of a gene-coding region that usually encodes for antibiotic resistance genes, such as ß-lactamase and acetyl transferases. Gene cassettes may also encode for genes that protect bacteria against the action of heavy metals and quaternary ammonium compounds (11, 21). A non-encoding region found in gene cassettes has a recombination site known as the 59-base element. Gene cassettes can be found as free circular DNA, but in most cases they are inside an integron.

At least four classes of integrons have been classified according to their integrase gene (25). Class 1 integrons are commonly found in clinical and non-clinical *E. coli* isolates (17), whereas Class 2 integrons have been reported at lower frequencies (19). The remaining two classes of integrons are rarely found. For example, during a six year survey in China, Class 1 integrons were found in 85.6% of the 111 analyzed isolates, while Class 2 integrons were found in 3.6% of the isolates. Class 3 integrons were not found in any of the isolates (27).

Antibiotic resistance has been found in urban centers as well as in animal production farms where excessive antibiotic consumption favors its appearance (2; 13, 13). In addition to urban centers, recent reports indicate that a large number of antibiotic resistant genes can also be found in the remote communities of Bolivia (30). In the present study, we investigated the presence and dissemination of antibiotic resistance genes in 250 samples of *E. coli* isolates collected from fecal samples in two remote communities in Ecuador. Despite the limited access to antibiotics in these communities, 32% of the samples collected carried antibiotic resistance genes and 11% showed evidence of horizontal gene transfer.

2. OBJECTIVES

2.1. General Objective

To investigate the occurrence of horizontal gene transfer (HGT) in commensal *E. coli* in two remote populations located in the northern coast of Ecuador.

2.2. Specific objectives

- To determine antimocrobial susceptibility of commensal *E. coli* in two remote populations.

- To assess clonal expansion of antibiotic resistant *E. coli*.

- To identify events of horizontal gene transfer in resistant isolates of commensal *E. coli*.

3. MATERIALS AND METHODS

3.1. Determination of resistant isolates.

3.1.1. Study Design.

This study was part of a bigger project that investigated the occurrence of diarrhea in the proximity of a new road built in Ecuador between 1996 and 2001 (10). Briefly, 22 rural communities from northern coastal Ecuador were visited 4 times since 2004. During each visit that lasted 15 days, three communities were visited and had human diarrhea cases collected every morning. Diarrhea cases were defined according to the criteria of the World Health Organization. For each case of diarrhea, two controls were randomly sampled from healthy individuals of the community and an additional control was randomly sampled from a healthy individual of the case household. Exclusively for this study, two communities from northern coastal Ecuador were visited between May and July 2006. The first called San Agustín is located along the new road built seven years ago and the other called Playa de Oro still has access only by boat. Each community had samples collected for a 15 day period, during which the same protocol for fecal sample collection was followed and no repeated samples were allowed.

We used 56 fecal samples obtained from individuals living in these two remote communities; 15 were diarrhea cases, 11 were controls from a healthy individual of the case household, and 30 were controls from healthy people of the community. San Agustín has 272 and Playa de Oro has 226 inhabitants. The majority of these individuals are

African-Ecuadorians. Informed consent was obtained for the collection of these samples and the protocol was approved by the IRB committees from University of Michigan and Universidad San Francisco de Quito.

3.1.2. Bacterial isolates.

The fecal samples were cultured in MacConkey agar for 24 hours at 37°C. Five lactose-fermenting colonies per fecal sample were inoculated on to Cromocult® agar plates (Merk, Germany) to identify *E. coli* isolates. A total of 279 isolates were obtained. *E. coli* was detected in 250 isolates. Taxonomic identification of the antibiotic resistant isolates was confirmed by API-E test (bioMérieux ® sa, France).

3.1.3. Antimicrobial susceptibility testing.

Antimicrobial susceptibilities were assessed by the Kirby-Bauer method in Mueller Hinton agar plates as recommended by the National Committee for Clinical Laboratory Standards (7). We used the following standard antibiotic discs: ampicillin (10 µg), tetracycline (30 g), trimethoprim/sulfamethoxazole (25 µg), gentamicin (10 µg), chloramphenicol (30 g), cefotaxime (30 g), and ciprofloxacin (5 µg). *E. coli* ATCC 25922 was used as a control. Isolates with resistance to ampicillin, tetracycline and trimethoprim/sulfamethoxazole (ATSr) were selected for further analysis. These isolates came from 4 diarrhea cases, 1 control from the case household, and 4 controls from healthy individuals of the community.

Descriptive statistic analysis was carried out in Stat View Version 5.0 software to find the resistance rate in the *E. coli* isolates, and to find the frequency of each resistance pattern encountered in this bacteria population. The table of frequencies generated by Stat View software was used to create a pie chart in Microsoft Excel.

3.2. Determination of clonal expansion in ATSr resistant isolates.

3.2.1. Pulsed Field Gel Electrophoresis (PFGE) typing.

This technique is the gold standard for DNA fingerprinting. All ATSr isolates were subjected to pulsed field gel electrophoresis (29). Briefly, cell cultures were diluted in a 200μl cell suspension buffer (75mM NaCl, 25mM EDTA, pH 8.0) to an OD_{610} of approximately 0.7, corresponding to a McFarland scale number 5. A volume of 10μl Proteinase K (20mg/ml) (New England Biolabs, Boston, MA), 1% SDS, and 200 μl of 1% agarose (Pulsed Field certified agarose, Bio Rad Laboratories, Hercules, CA) was added and dispensed into plug molds which were transferred to 1ml of lysis buffer (50mM Tris-HCl, 50mM EDTA, pH 8.0, 1% N-laurylsarcosine, and 0.1mg/ml of Proteinase K) and incubated for overnight (12-18 h) at 54°C. Plugs were washed 4 times with a 30ml warm (50°C) TE buffer (10mM Tris, 1mM EDTA, pH 8.0). Genomic DNA inside the plugs was digested with 60 U of *Xba*I (New England Biolabs, Boston, MA) overnight at 37°C.

Restriction fragments were separated on 150 ml of 1% agarose (Bio-Rad Laboratories) gel using a CHEF electrophoresis cell (Bio-Rad Laboratories) in a 3L of

0.5x TBE buffer with 3ml of 100μM thiourea (29). The conditions for PFGE were 13°C

at 6 V/cm for 22 hours with an increasing pulse time of 2.2 - 54.2 s. Gels were stained

with ethidium bromide, and visualized with UV light. The band pattern of PFGE gels was

analyzed by the software GelCompar using the Pearson correlation to determine if there

was a relation between the isolates genome.

3.2.2. DNA extraction.

DNA from *E. coli* isolates was necessary for subsequent PCR reactions. It was

extracted using DNAzol® reagent (Invitrogen, Carlsbad, CA) as specified by the

manufacturer. Briefly, a cell suspension was lysed with 1ml of DNAzol® reagent and

100% ethanol was added. The precipitated DNA was then cleaned with 300 μl of 75%

ethanol and DNA was resuspended in a TE buffer. DNA quantification was not

necessary.

3.2.3. ERIC PCR typing.

To assess the variability of *E. coli* clones found in an individual's luminal

microbiota, it was necessary to do fingerprinting, but with a technique that could let us

work with more samples in one experiment. Fingerprints from the 250 *E. coli* isolates

were obtained by amplifying ERIC sequences (25). Primer ERIC 2

(5'AAGTAAGTGACTGGGGTGAGCG-3') was used for PCR amplifications. Strain *E.*

coli ATCC 25922 was used as a positive control and sterile water was used as a negative

control. A 2 μl aliquot of DNA suspension was amplified with Pure Taq Ready-To-Go

PCR beads (Amersham Biosciences, Piscataway, NJ). The PCR conditions were 94°C for 2 min; 35 cycles of 94°C for 30s, 60°C for 1 min, 72°C for 4.5min, and a final extension at 72°C for 1 min. Twenty ERIC PCR fingerprinting profiles were confirmed by PFGE that is the gold standard. Banding profile was analyzed from each ERIC PCR belonging to an *E. coli* isolate. These profiles were compared visually between the five *E. coli* isolates from an individual to determine the number of clones encountered. The average of *E. coli* clones found in each individual was calculated by the arithmetic mean of the number of *E. coli* clones that were found in each individual.

3.3. Determination of horizontal gene transfer (HGT) events in ATSr resistant isolates.

3.3.1. Integron identification.

Since integrons are contained in conjugal plasmids that are the common mobile for HGT, it was necessary to characterize the presence of integrons in our resistant sample (ATSr). Class 1 and class 2 integron variable sequences were amplified from ATSr strains as described elsewhere (32). Briefly, primers hep58 (5'-TCATGGCTTGTTATGACTGT-3') and hep59 (5'-GTAGGGCTTATTATGCACGC-3') amplified the cassette region of class 1 integrons. Primers hep51 (5'-GATGCCATCGCAAGTACGAG-3') and hep74 (5'-CGGGATCCCGGACGGCATGCACGATTTGTA-3') amplified the cassette region of class 2 integrons. Strains of *E. coli* with the genes *dfrA5* (class 1 integron) and *dfrA12* (class 2 integron) were used as positive controls and the strain *E. coli* K12 was used as a

negative control. A 1 µl aliquot of DNA suspension was amplified with 0.4 µM primers, 1x PCR buffer, 0.2mM dNTPs, 0.90 µM MgCl$_2$, and 2.5 U of Taq polymerase (Invitrogen, Brazil). PCR conditions were: 94°C for 30s, followed by 30 cycles of: 94° for 30s; 57°C for 30s; and 72°C for 12 min, and a final extension of 72°C for 10 min. Gels were stained with ethidium bromide and visualized with UV light. Descriptive statistics of positive integron amplification were calculated by counting the frequency of DNA samples that amplify for Class 1 and Class 2 integrons.

Integron amplicons were classified into four arbitrary groups according to the amplifying primers and their size. Groups A-C belonged to Class 1 integrons, while Group D belonged to Class 2 integrons. Group A corresponded to 2036bp approximately, group B corresponded to 1700bp approximately, group C corresponded to 850bp approximately, and group D corresponded to 1330bp approximately.

We modified the restriction fragment length polymorphism (RFLP) procedure described by Leverstein-van Hall et al. (2002) (16) and used this protocol to characterize the same-size amplicons to verify that they were the same. Three enzymes were tested: *Pst*I, *Pvu*II, and *Hind*III, but only two worked. Group A amplicons could be digested only with *Hind*III whereas group B amplicons could be digested only with *Pvu*II. Results were visualized in ethidium bromide stained gels. Group C generated a PCR fragment size already distinguishable from groups A and B. Therefore no further procedure as a digestion was necessary to be done with this group. Group D did not need to be digested because they were the same clone and they amplified for integron Class 2.

3.3.2. β-lactamase genes identification and sequencing.

β-lactamases are enzymes that inactivate penicillin and/or cephalosporins. β-lactamases classification is complex, but plasmidic enzymes TEM-1, TEM-2, and SHV-1 (produced by genes bla_{TEM} and bla_{SHV}) are found to be common causes of resistance to ampicillin in Enterobacteriaceae. For this reason, β-lactamases bla_{TEM}, and bla_{SHV} genes were amplified from the eighteen ATSr isolates as described elsewhere (24). Primers TEM-F (5'-TCGGGGAAATGTGCGCG-3') and TEM-R (5'-TGCTTAATCAGTGAGGCACC-3') amplified bla_{TEM} gene. Primers SHV-F (5'-CACTCAAGGATGTATTGTG-3') and SHV-R (5'-TTAGCGTTGCCAGTGCTCG-3') were used to amplify bla_{SHV}. The strain *E. coli* ER2420 with pACYC177 was used as a positive control for bla_{TEM} gene, and *E. coli* K12 was used as a negative control. A 2 μl aliquiot of DNA suspension was amplified with Pure Taq Ready-To-Go PCR beads (Amersham Biosciences, Piscataway, NJ). The PCR conditions were: 96°C for 15s; 24 cycles of 96°C for 15s, 52°C for 15s, and 72°C for 2 min. Gels were stained with ethidium bromide and visualized with UV light. No amplification for bla_{SHV} gene was detected even after several attempts.

Sequencing of PCR products was carried out at the University of Michigan. The raw chromatographs were read, and the sequences from both directions were assembled into one manually. Low quality sequences from the ends were removed. Sequences were aligned by ClustalW in Mega 3.1 software. Nucleotide sequences of β-lactamase (bla_{TEM})

genes were BLASTed against sequences in GenBank and analyzed with Mega 3.1 software using Neighbor Joining method.

Accession numbers of the sequences described in this manuscript are: EU352887 (isolate 51A), EU352888 (isolate 51B), EU352889 (isolate 51C), EU352890 (isolate 51D), EU352891 (isolate 51E), EU352892 (isolate 52D), EU352893 (transconjugate of isolate 52D), EU352894 (isolate 80D), EU352895 (isolate 81A), EU352896 (transconjugant of isolate 81A), EU352897 (isolate 97B), EU352898 (isolate 73B), EU352899 (isolate 75E), EU352900 (isolate 15A), EU352901 (isolate 15E), EU352902 (isolate 15B), EU352903 (isolate 15C), EU352904 (isolate 36B), EU352905 (transconjugant of isolate 36B), EU352906 (isolate 36C), and EU352907 (isolate 36D).

3.4. Determination of conjugal capability in ATSr resistant isolates.

3.4.1. Mutation of a K12 *E. coli* strain.

To select transconjugants isolates from conjugation experiments, it was necessary to create a mutant strain. A K12 *E. coli* strain provided by Lee Riley (University of California, Berkeley, CA) was used to select a nalidixic acid resistant mutant, creating a K12 strain (NaAc^r). A 100 ml Brain Heart Infusion was inoculated with *E. coli* K12 and cultured for 20 hours at 37°C. Next, a 100 ml of sterile media containing 50 µg/ml of nalidixic acid (final concentration of 25 µg/ml) was added to the culture. After an additional 24 hours of incubation at 37°C, 200 µl of the K12 was then cultured on a Triptic Soy Broth agar plate containing an additional 25 µg/ml of nalidixic acid. To

assess the isogenic nature of the mutant and of the K12 strains, both strains were
subjected to ERIC-PCR analysis (28).

3.4.2. Conjugation experiments.

Conjugative transfer of resistance plasmids was tested by mixing the cultures of
E. coli K12 (NaAc^r) and an antibiotic resistant isolate (13) in a proportion of 1:1.
Transconjugants were selected by transferring the E.coli cultures to Triptic Soy Broth
agar plates with tetracycline (12μg/ml) and nalidixic acid (25 μg/ml) after 24 hours of
incubation. The antimicrobial susceptibilities of the transconjugants were established by
the Kirby Bauer method (7). No descriptive statistics were necessary since all the isolates
were capable of transferring resistance by conjugation.

4. RESULTS

4.1. Resistant profiles of isolates.

Of the 250 isolates of *E. coli*, 80 (32%) were resistant at least to one antibiotic, the remaining 170 (68%) were susceptible to all antibiotics tested. Combined resistance to ampicillin, tetracycline, and trimethoprim/sulfamethoxazole (ATSr) was the most prevalent multiresistance pattern and accounted for 23% of the resistant isolates (N = 18) (Figure 1). Table 1 describes each of these 18 isolates. Eleven resistant isolates (14%) showed resistance to more than 5 antibiotics, whereas 51 resistant isolates (64%) showed resistance to one or two antibiotics only (Figure 1). The 18 isolates that were ATSr resistant were further characterized for clonality and HGT.

4.2. Clonal relationships using Pulsed Field Gel Electrophoresis (PFGE).

A total of 9 PFGE profiles were identified among the 18 ATSr isolates (Figure 2 and Table 1). As shown in Table 1, all five isolates from individual 1946 were classified as clonal. This was also true for the three isolates from individual 1961. Isolates from individual 2077 were classified into two clones. The remaining individuals had only one ATSr isolate each. Except for profile 7, which was found in two isolates from two different individuals (1961 and 1962) living in the same community (Table 1 and Figure 2), all profiles were unique to specific individuals.

4.3. Integron RFLP analysis.

Class 1 integrons were observed in 12 of the 18 (67%) ATSr isolates, Class 2 integrons were observed in 2 of 18 (11%) isolates (Figure 3), and no Class 1 nor Class 2 integrons were observed in 4 of the 18 (22%) of these isolates. As expected for a variable region, different sizes of integrons were observed (Figure 3). Integron RFLP of groups A and B are shown in (Figure 4a-b).

Of the 18 isolates, 13 (72%) integron RFLPs correlate with genomic PFGE profiles analysis (Table 1). For example, three isolates from individual 1946 were of RLFP group A and of PFGE profile 8 whereas six isolates from individuals 1961 and 1962 were of RLFP group B and of PFGE profile 7. Two isolates (80D and 97B) presented the same integron RFLP as isolates 51A to 51E and 52D. However, the two isolates had different PFGE profiles.

4.4. β-lactamase gene analysis.

All the eighteen DNA samples of these isolates amplified the bla_{TEM} gene, but none amplified the bla_{SHV} gene, for this reason no further analysis was necessary with bla_{SHV} gene. According to their sequences, they all clustered in the group of bla_{TEM-1} gene (Figure 5). The bla_{TEM} gene sequences were classified into three arbitrary clusters: X, Y, Z (Figure 6 and Table 1).

At least 8 of 18 (44%) DNA sequence clusters were in agreement with the RLFP classification. All 8 isolates with integrons RFLP profile B contain β-lactamase gene

sequence cluster X. Two isolates from individual 2077 showed genes in cluster Y and had integrons with RLFP profile D. However, two other strains obtained from the same individual carrying unidentified integron, also contained the bla_{TEM} gene belonging to cluster Y. The same cassette was also observed in three other isolates, namely isolate 73B from individual 1983, isolate 75E from individual 1985, and in isolate 36B from individual 1946 (Table 1).

Taken together, three isolates show the same integron (51A-51E and 52D -that constitutes a single clone-, 80D, and 97B), and six isolates (81A, 73B, 75E, 36B, clones 15A and 15E, clones 15B and 15C) share its allele with at least another isolate. Despite the fact that these isolates share the same alleles, they do not carry the same integrons.

4.5. Horizontal gene transfer by conjugation.

All ATSr isolates were able to transfer their resistance to the K12 NaAc^r receptor strain. The presence of integrons was verified in three transconjugants in which amplicons had the same size as the amplicons of their donors and their RLFP was the same (Figure 3). Similarly, the β-lactamase sequences in donors and transconjugants were verified in ATSr strains 36B, 52D, and 81A.

5. DISCUSSION

In this study we found indication for the occurrence of horizontal gene transfer in nine (50%) of the isolates we tested. This finding is in agreement with previous reports that suggested a high contribution of lateral transfer in the dissemination of antibiotic resistance in *E. coli* (1, 3, 27). The HGT occurs at two levels: integron transfer and cassette exchange. It was observed the same integron RFLP profile and β-lactamase gene sequence in isolates from individuals 1961/1962 (the same clone), 1990, and 2007 which shows HGT of the same genetic structure between individuals of the same community. The present report also presents evidence of only cassette exchange among *E. coli* isolates such as isolates from individuals carrying group Y β-lactamases that had different PFGE profile and different integron. This phenomenon has not been described in this taxon before, but it has been suggested to occur in integrons of *Pseudomonas* (12).

The presence of the same β-lactamase cassette in two distinct isolates of *E. coli* found in individual 2077 may provide evidence of horizontal gene transfer within the human intestine. Interestingly, this cassette is present in 5 distinct PFGE profile isolates (1, 5, 6, and 8). A previous report has shown that antibiotic resistance gene transfer can occur under simulated intestinal conditions (4).

In this study it was shown that Class 1 integron was the main structure associated to antibiotic resistance in commensal *E. coli*. This finding corroborates that class 1 integron is a common structure associated to clinical and community *E. coli* isolates (27). Four ATSr strains did not seem to contain integron class 1 not class 2, but they did

contain β-lactamase gene which was transferable by conjugation. These data may indicate that these isolates contained another type of integron or that the cassette has been inserted in a plasmid without integron which has been described previously (25).

The presence of such a high prevalence of antibiotic resistant strains in remote communities (32% showed antibiotic resistance) was unexpected, although 67% of antibiotic resistant *E. coli* have been reported in remote communities of Bolivia (22). This phenomenon may be related to the fact that antibiotic resistance is mediated by Class 1 integrons which in its basic structure, it carries antibiotic resistance cassettes along with genes coding resistance to heavy metals and other environmental harmful compounds (11, 21). The presence of naturally occurring antibiotics (8) or toxic substances in the environment will select bacteria carrying multiresistance.

Environmental conditions such as naturally occurring antibiotics could constitute a primary selection of resistance (22), however, DNA sequence evidence does not support this hypothesis (1). In addition, antibiotic resistance could enter a remote community through the introduction of occasional travelers and animals that carry resistant strains and spread in the community. In this case, it would be expected a circulation of a limited number of clones or mobile genetic elements that carry common genes of antibiotic exposed communities. Nevertheless, the mechanisms that cause a high prevalence of antibiotic resistance in communities that have a limited access to this kind of medication continue unclear (22). It would be interesting to find out if some antibiotic resistance genes are transferred from environmental sources.

Remote communities may be ideal places to study horizontal transfer of antibiotic genes because of the apparent low number of *E. coli* clones. In 250 isolates from 50 individuals we found and average of 1.6 clones (range, 1 to 4 clones/sample) per individual. Studies carried out with stools of young girls in the US found an average of 2 (range, 1 to 8 clones/sample) clones per fecal sample (26).

Horizontal transfer of genes is a dynamic molecular process that creates diversity in bacteria. In this case, it creates more combinations of resistance genes alleles. As a consequence, antibiotic resistance is destined to increase and it is going to be difficult to eradicate it from the environment. Moreover, unexpected resistance to synthetic compounds such as fluoroquinolones is circulating in bacteria population (18). It is possible that antibiotic usage cannot be the only reason for selecting resistant phenotypes, since they are also found in locations with little or no access to antibiotics.

REFERENCES

Aminov, R.I., and R.I. Mackie. 2007. Evolution and ecology of antibiotic resistance genes. FEMS Microbiol. Lett. **271:** 147-161.

Austin D.J., K.G. Kristinsson, and R.M. Anderson.1999. The Relationship between the volume of antimicrobial consumption in human communities and the frequency of resistance. Proc. Natl. Acad. Sci. USA **96:** 1152-56.

Blahna, M.T., C.A Zalewski, J. Reuer, G. Kahlmeter, B. Foxman, and C.F. Marrs. 2006. The role of horizontal gene transfer in the spread of trimethoprim-sulfamethoxazole resistance among uropathogenic *Escherichia coli* in Europe and Canadá. J. Antimicrob. Chemother. **57:** 666-672.

Blake D.P., K. Hillman, D.R. Fenlon and J.C. Low. 2003. Low Transfer of antibiotic resistance between commensal and pathogenic members of the Enterobacteriaceae under Ileal Conditions. J. Applied Microbiol. **95:** 428–436.

Bradford, P.A. 2001. Extended-Spectrum β-Lactamases in the 21th Century: Characterization, Epidemiology, and Detection of this Important Resistance Threat. Clin. Microbiol. Rev. **14:** 933-951.

Briñas, L., M. Zarazaga, Y. Sáenz, F. Ruiz-Larrea, and C. Torres. 2002. β-Lactamases in Ampicillin-Resistant *Escherichia coli* Isolates from Food, Humans, and Healthy Animals. Antimicrob. Agents Chemother. **46:** 3156-3163.

Cortez, J.H. 2005. Sección II, Capítulo 4. Prueba de Difusión por Disco. *In* M.B. Coyle (ed.), Manual de Pruebas de Susceptibilidad Antimicrobiana. Seattle. http://www.ops-oms.org/spanish/ad/ths/ev/labs_sucep_antimicro.pdf

D'Costa, V.M., K.M. McGrann, D.W. Hughes, and G.D. Wright. 2006. Sampling the Antibiotic Resistome. Science. **311**: 374-377.

Eckburg, P.B., E.M. Bik, C.N. Bernstein, E. Purdom, L. Dethlefsen, M. Sargent, S.R. Gill, K.E. Nelson, and D.A. Relman. 2005. Diversity of the Human Intestinal Microbial Flora. Science. **308**: 1635-1638.

Eisenberg, J.N.S., W, Cevallos, K. Ponce, K. Levy, S.J. Bates, J.C. Scott, A. Hubbard, N. Vieira, P. Endara, M. Espinel, G. Trueba, L.W. Riley, and J. Trostle. 2006. Environmental change and infectious disease: How new roads affect the transmission of diarrheal pathogens in rural Ecuador. Proc. Natl. Acad. Sci. USA **103:** 19460-19465.

Gaze, W.H., N. Abdouslam, P.M. Hawkey, and E.M.H.Wellington. 2005. Incidence of Class 1 Integrons in a Quaternary Ammonium Compound-Polluted Environment. Antimicrob. Agents Chemother. **49:**1802-1807.

Giske, C.G., B. Libisch, C. Colinon, E. Scoulica, L. Pagani, M. Füzi, G. Kronvall, and G.M. Rossolini. 2006. Establishing Clonal Relationships between VIM-1-Like Metallo-β-Lactamases-Producing *Pseudomonas aeruginosa* Strains from Four European Countries by Multilocus Sequence Typing. J. Clin. Microbiol. **44:** 4309-4315.

Kang, H.Y., Y.S. Jeong, J.Y. Oh, S.H. Tae, C.H. Choi, D.C. Moon, W.K. Lee, Y.C. Lee, S.Y. Seol, D.T.Cho, and J.C. Lee. 2005. Characterization of antimicrobial resistance and class 1 integrons found in *Escherichia coli* isolates from humans and animals in Korea. J. Antimicrob. Chemother. **55**: 639-644.

Kruse, H., and H. Sŏrum. 1994. Transfer of Multiple Drug Resistance Plasmids between Bacteria of Diverse Origins in Natural Microenvironments. Appl. Environ. Microbiol. **60:** 4015-4021.

Lawrence, G.L., and H. Ochman. 1998. Molecular archaeology of the *Escherichia coli* genome. Proc. Natl. Acad. Sci. USA. **95:** 9413-9417.

Leverstein-van Hall, M., A.T.A. Box, H.E.M. Blok, A. Paauw, A.C. Fluit, and J. Verhoef. 2002. Evidence of Extensive Interspecies Transfer of Integron-Mediated Antimicrobial Resistance Genes among Multidrug-Resistant Enterobacteriaceae in Clinical Setting. J. Infect. Dis. **186**: 49-56.

Leverstein-van Hall, M., H.E.M. Blok, R.T. Dongers, A. Paauw, A.C. Fluit, and J. Verhoef. 2003. Multidrug Resistance among Enterobacteriaceae Is Strongly Associated with the Presence of Integrons and Is Independent of Species or Isolate Origin. J. Infect. Dis. **187:** 251-259.

Livermore, D. (2007). The zeitgeist of resistance. J. Antimicrob. Chemother. **60**: *Suppl.* 1, i59-i61.

Machado, E., R. Cantón, F. Baquero, J.C. Galán, A. Rollán, L. Peixe, and T.M. Coque. 2005. Integron Content of Extended-Spectrum-β-Lactamases-Producing *Escherichia coli* Strains over 12 Years in a Single Hospital in Madrid, Spain. Antimicrob. Agents Chemother. **49:** 1823-1829.

Martínez, J.L., F. Baquero. 2002. Interactions among Strategies Associated with Bacterial Infection: Pathogenicity, Epidemicity, and Antibiotic Resistance. Clin. Microbiol. Rev. **15 :** 647-679.

Nemergut, D.R., A.P. Martin, and S.K. Schmidt. Integron Diversity in Heavy-Metal-Contaminated Mine Tailings and Inferences about Integron Evolution. Appl. Environ. Microbiol. **70 :** 1160-1168.

Pallecchi, L., C. Lucchetti, A. Bartoloni, F. Bartalesi, A. Mantella, H. Gamboa, A. Carattoli, F. Paradisi, and G.M. Rossolini. 2007. Population Structure and Resistance Genes in Antibiotic-Resistant Bacteria from Remote Community with Minimal Antibiotic Exposure. Antimicrob. Agents Chemother. **51**: 1179-1184.

Pál, C., B. Papp, and M.J. Lercher. 2005. Adaptative evolution of bacterial metabolic networks by horizontal gene transfer. Nat. Genet. **37;** 1372-1375.

Pitout, J.D.D., K.S. Thomson, N.D. Hanson, A.F. Ehrhardt, E.S. Moland, and C.C. Sanders. 1998. B-Lactamases Responsible for Resistance to Expanded-Spectrum Cephalosporins in *K. pneumoniae*, *E .coli*, and *P. mirabilis* Isolates Recovered In South Africa. Antimicrob. Agents Chemother. **42:** 1350-1354.

Recchia, G.D., R.M. Hall. 1995. Plasmid evolution by acquisition of mobile gene cassettes: plasmid pIE723 contains the *aadB* gene cassette precisely inserted at a secondary site in the IncQ plasmid RSF1010. Mol. Microbiol. **15:** 179-187.

Schlager, T.A., J.O. Hendley, A.L. Bell, and T.S. Whittam. 2002. Clonal Diversity of *Escherichia coli* Colonizing Stools and Urinary Tracts of Young Girls. Infect. Immun. **70:** 1225-1229.

Su, J., L. Shi, L. Yang, Z. Xiao, X. Li, and S. Yamasaki. 2006**.** Analysis of integrons in clinical isolates of *Escherichia coli* in China during the last six years. FEMS Microbiol. Lett. **254:** 75-80.

Versalovic, J., T. Koeuth, and J.R. Lupski. 1991**.** Distribution of repetitive DNA sequences in eubacteria and application to fingerprinting of bacterial genomes. Nucleic Acids Res. **19:** 6823-6831.

Vieira, N. S.J. Bates,, O.D. Solberg, K. Ponce. R. Howsmon, W. Cevallos, G. Trueba L. Riley, and J.N.S. Eisenberg. 2007**.** High prevalence of enteroinvasive *Escherichia coli* isolated in a remote region of northern coastal Ecuador. Am. J. Trop. Med. Hyg. **76:** 528-533.

Walson J.L., B. Marshal, M.M. Pokhrel, K.K. Kafle, and S.B. Levy. 2001. Carriage of Antibiotic-Resistance Fecal Bacteria in Nepal Reflects Proximity of Kathmandu. J. Infect. Dis. **184:** 1163-1169.

Welch, R.A., V. Burland, G. Plunkett III, P. Redford, P. Roesch, D. Rasko, E.L. Buckles, S.-R. Liou, A. Boutin, J. Hackett, D. Stroud, G.F. Mayhew, D.J. Rose, S. Zhou, D.C. Schwarts, N.T. Perna, H.L.T. Mobley, M.S. Donnenberg, and F.R. Blattner. 2002**.** Extensive mosaic structure revealed by the complete genome sequence of uropathogenic *Escherichia coli*. Proc. Natl. Acad. Sci. USA. **99:** 17020-17024.

White, P.A., C.J. McIver, Y. Deng, and W.D. Rawlinson. 2000**.** Characterisation of two new gene cassettes, *aadA5* and *dfrA17*. FEMS Microbiol. Lett. **182**: 265-269.

Xu J, M.A. Mahowald, R.E. Ley, C.A. Lozupone, M. Hamady, E.C. Martens, B. Henrissat, P.M. Coutinho, P. Minx, P. Latreille, H. Cordum, A. Van Brunt, K. Kim, R.S. Fulton, L.A. Fulton, S.W. Clifton, R.K. Wilson, R.D. Knight, and J.I. Gordon. 2007**.** Evolution of Symbiotic Bacteria in the Distal Human Intestine. PLoS Biol. **5:** 1574-1586.

TABLE 1. Information on eighteen isolates resistant to ampicillin, tetracycline and trimethoprim sulfamethoxazole. The first three colums identify the person community and isolate. The last three columns provide molecular information of the isolate. The PFGE profile corresponds to the classification in Figure 2. .Integron RFLP: classification of integrons encountered within the isolates; integron RFLP A and B were obtained with *Hind*III and *Pvu*II, respectively; group D were integrons class 2; group NI were isolates that didn't amplify for class 1 nor class 2 integrons. The *bla* gene cluster number corresponds to the classification shown in Figure 6.

Individual number	Community name	Isolate name	PFGE profile	Integron RFLP	bla_{TEM} gene cluster
1946	San Agustín	36B	8	A	Y
	San Agustín	36C	8	A	Z
	San Agustín	36D	8	A	Z
1961	San Agustín	51A	7	B	X
	San Agustín	51B	7	B	X
	San Agustín	51C	7	B	X
	San Agustín	51D	7	B	X
	San Agustín	51E	7	B	X
1962	San Agustín	52D	7	B	X
1983	San Agustín	73B	5	C	Y
1985	San Agustín	75E	1	NI	Y
1990	San Agustín	80D	9	B	X
1991	San Agustín	81A	3	NI	X
2007	San Agustín	97B	4	B	X
2077	Playa de Oro	15A	2	NI	Y
	Playa de Oro	15B	6	D	Y
	Playa de Oro	15C	6	D	Y
	Playa de Oro	15E	2	NI	Y

Abbreviations: NI, not identified

FIGURE 1. Resistance pattern found in E. coli isolates. Resistances to: AMP (ampicillin), CIP (ciprofloxacin), CHL (chloramphenicol), GE (gentamicin), SXT (trimethoprim/sulfamethoxazole), TET (tetracycline).
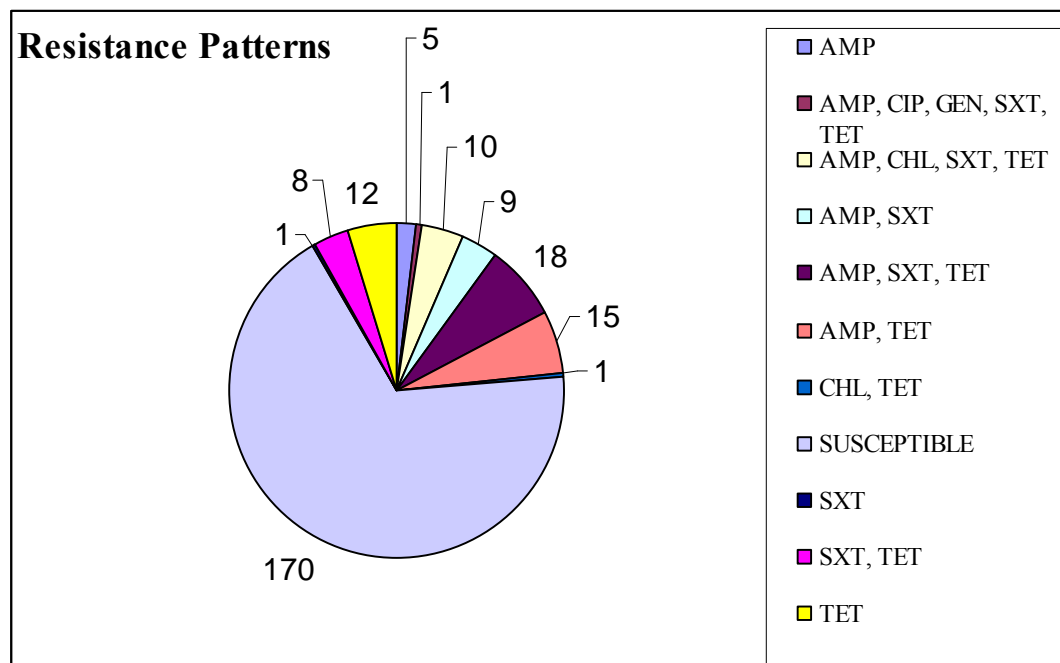
FIGURE 2. PFGE of the eighteen isolates resistant to ampicillin, tetracycline, and trimethoprim/sulfamethoxazole. Clonal relationships are observed between isolates 36B, 36C, and 36D belonging to individual 1946, between isolates 51A, 51B, 51C, 51D, 51E and 52D belonging to individuals 1961 and 1962, between isolates 15A and 15E, and between isolates 15B and 15C belonging to individual 2077. The rest of strains are not related to any other strain.
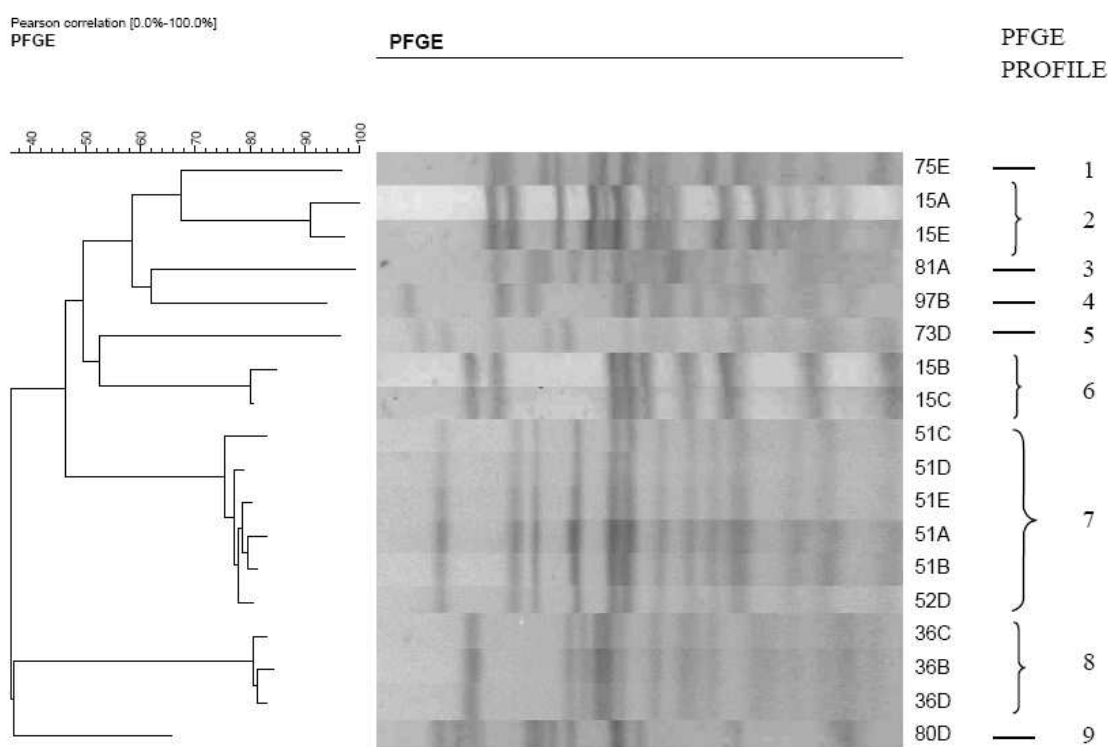
FIGURE 3. Amplification of Class 1 and 2 integrons. Lane 1: Standard 1 kilobase, Lane 2: Positive control of integron class 1, Lane 3: negative control, Lane 4: isolate 51A, Lane 5: isolate 51B, Lane 6: isolate 51D, Lane 7: isolate 36B, Lane 8: isolate 51C, Lane 9: transconjugant 51C, Lane 10: isolate 73B, Lane 11: isolate 36C, Lane 12: transconjugant 73B, Lane 13: isolate 51E, Lane 14: isolate 36D, Lane 15: isolate 52D, Lane 16: isolate 80D, Lane 17: isolate 97B, Lane 18: transconjugant 97B, Lane 19: amplification of integron class 2 of isolate 15B, Lane 20: amplification of integron class 2 of isolate 15C. Letters down of numbers are the classification of integrons encountered within the isolates. Group A and B were digested with *Hind*III and *Pvu*II, respectively.
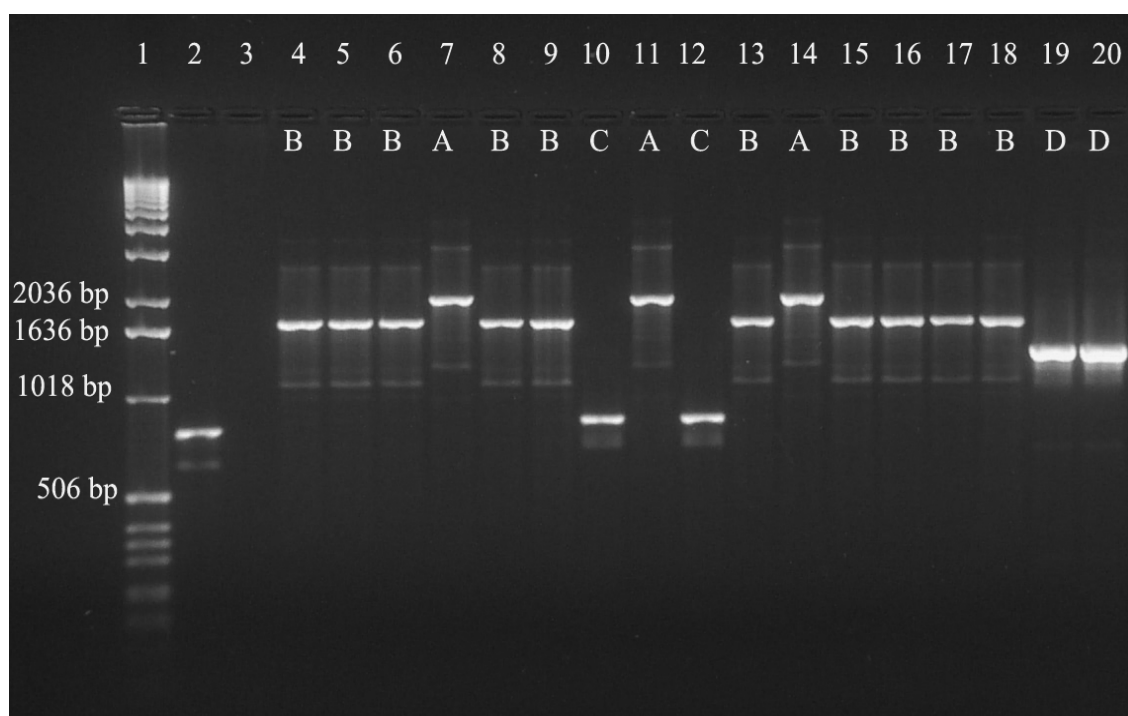
FIGURE 4. Integron RFLPs of group A and group B integrons. a) Integron RFLP of group A integron amplicons, digested with *Hind*III in: Lane 2, isolate 36B; Lane 3, isolate 36C; Lane 4, isolate 36D, Lane 1: control without enzyme digestion. b) Integron RFLP of group B integron amplicons, digested with *Pvu*II in: Lane 2, isolate 51A; Lane isolate 3, 51B; Lane 4, isolate 51C; Lane 5, transconjugate of isolate 51C; Lane 6, isolate 51D; Lane 7, isolate 51E; Lane 8, isolate 52D; Lane 9, isolate 80D; Lane 10, isolate 97B; Lane 11, transconjugate of isolate 97B; Lane 1, control without enzyme digestion.
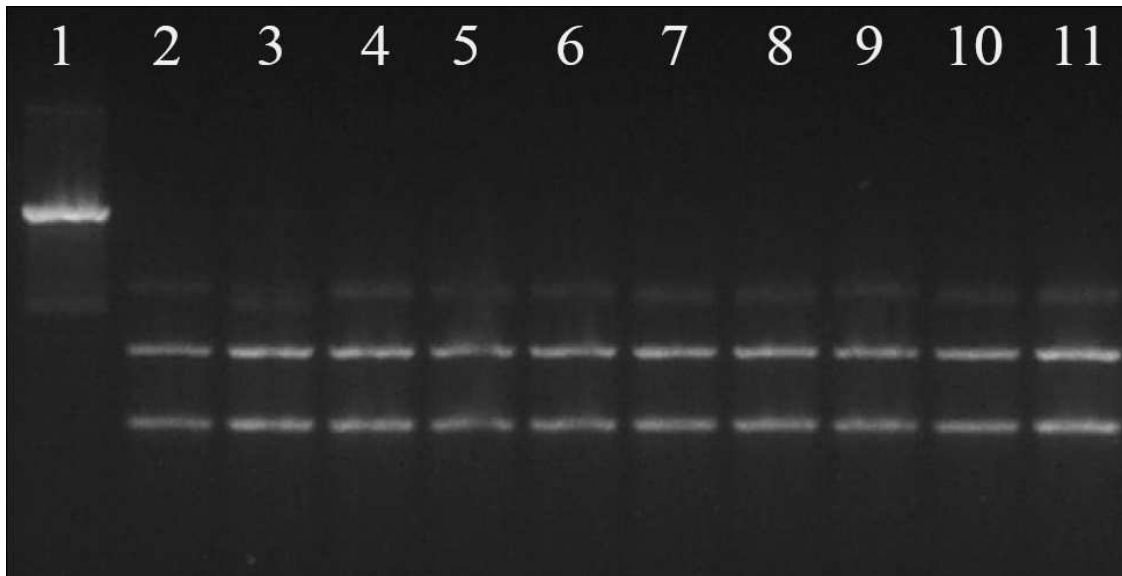
a)
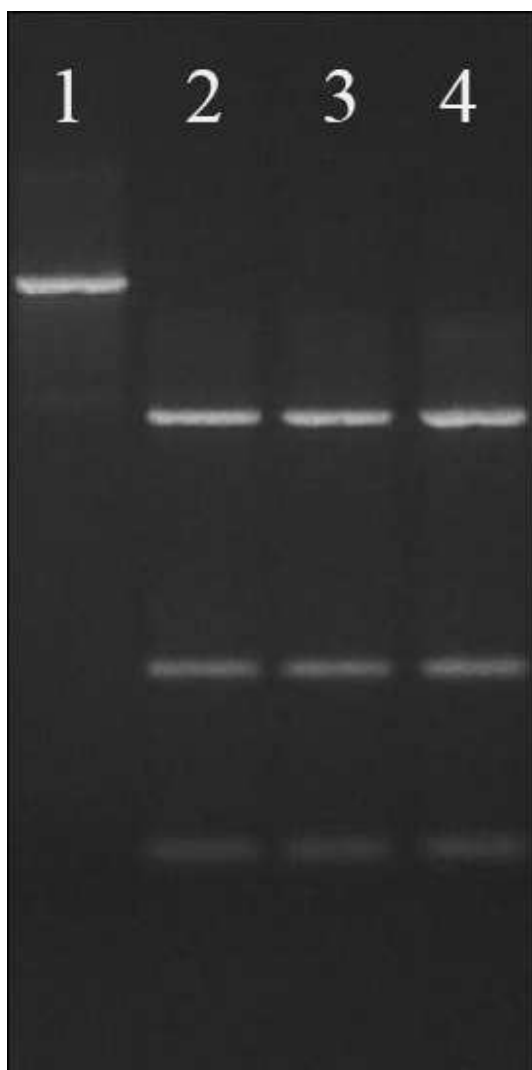
b)

FIGURE 5. Neighbor Joining analysis of beta-lactamase sequences of eighteen isolates

resistant to ampicillin, tetracycline and trimethoprim/sulfamethoxazole, and other

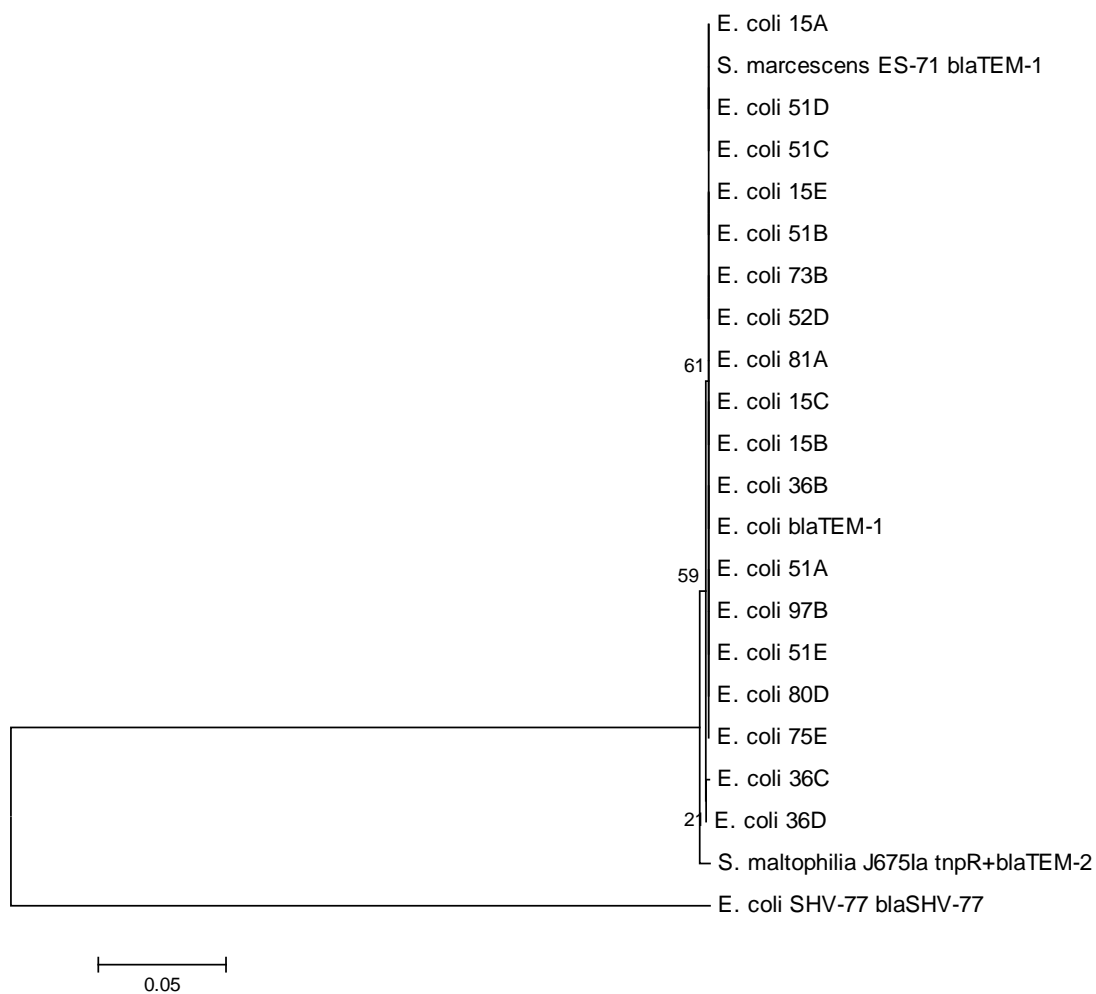blaTEM and blaSHV genes. β-lactamase SHV gene was the outgroup.

FIGURE 6. Neighbor joining analysis of β-lactamase sequences of eighteen isolates resistant to ampicillin, tetracycline and trimethoprim/sulfamethoxazole. Letters X, Y, and Z are arbitrary designated clusters of β-lactamase genes encountered in the ATSr isolates.