

Clonal Heterogeneity and Efficacy of BOX and (GTG)₅ Fingerprinting Methods for Molecular Typing of *Escherichia coli* Isolated from Chickens in IRI

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Abstract

This study evaluates the clonal heterogeneity and efficacy of BOX-PCR and (GTG)₅-PCR for DNA-based typing of *Escherichia coli* strains isolated from feces of chickens in IRI. Fecal samples were collected from chicken husbandry followed by *E. coli* isolation through biochemical tests. Isolates were finger printed by BOX-A1 and (GTG)₅ primers. Dendrograms were generated based on 80% similarity and Shannon-Weaver index was calculated. One hundred and six *E. coli* isolates were obtained from chicken's fecal sample. By (GTG)₅ primer, of 106 isolates, two isolates were untypeable, while 104 isolates generated 100 unique, and 2 duplicate profiles. The dendrogram generated six clusters (G1-G6). With BOX-PCR, 106 *E. coli* isolates revealed 50 unique BOX profiles, in addition to 22 repetitive profiles, while 12 isolates were untypeable. Based on the bands and dendrogram, the 106 strains were grouped into six clusters (B1-B6). Shannon-Weaver index was 4.665 for (GTG)₅-PCR and 0.281 for BOX-PCR. (GTG)₅-PCR revealed complex clonal heterogeneity, more discriminatory power, less untypeable isolates, higher Shannon-Weaver index, and less isolates with the same profile in comparison to BOX-PCR. Although (GTG)₅-PCR proved to be a powerful typing method, it is recommended to combine two or more different typing methods for higher discriminatory power.

Keywords: BOX-PCR, Chicken, Clonal heterogeneity, *Escherichia coli*, (GTG)₅-PCR, Molecular typing

BOX ve (GTG)₅ Parmak izi Metotlarının İran'da Tavuklardan İzole Edilen *Escherichia coli*'nin Moleküler Tiplendirilmesinde Klonal Heterojenite ve Yeterliliği

Özet

Bu çalışma ile İran'da tavuk dışkılarından izole edilen *Escherichia coli* suşlarının DNA temelli tiplendirmesinde BOX-PCR ve (GTG)₅-PCR metotlarının klonal heterojenitesi ve yeterliliği araştırılmıştır. Biyokimyasal test ile *E. coli* tespit edilen kümeslerden dışkı örnekleri toplandı. İzolatların parmakizi BOX-A1 ve (GTG)₅ primerler kullanılarak alındı. Dendrogramlar %80 benzerlik baz alınarak üretildi ve Shannon-Weaver endeksi hesaplandı. Yüzsekiz *E. coli* izolatu tavuk dışkı örneklerinden elde edildi. (GTG)₅ primer ile yapılan analizde 106 izolatu 2'si tiplendirilemezken 104 izolattan 100 özgün ve 2 duplike profil üretildi. Dendrogram altı küme (G1-G6) oluşturdu. BOX-PCR ile 106 *E. coli* izolatından 22 tekrarlayan profile ile birlikte 50 özgün BOX profili oluşurken 12 izolat tiplendirilemedi. Oluşan bantlar ve dendrograma göre 106 suş 6 küme (G1-G6) içerisinde gruplandırıldı. Shannon-Weaver endeksi (GTG)₅-PCR için 4.665, BOX-PCR için ise 0.281 olarak belirlendi. (GTG)₅-PCR; BOX-PCR ile karşılaştırıldığında kompleks klonal heterojenite, daha fazla ayırt edici güç, daha az tiplendirilemeyen izolat, daha yüksek Shannon-Weaver endeksi ve daha az aynı profilli izolat elde edilmesini sağladı. (GTG)₅-PCR daha güçlü tiplendirme metodu olmasına rağmen iki veya daha fazla tiplendirme metodunun birlikte kullanılması daha yüksek ayırıcı güç için önerilmektedir.

Anahtar sözcükler: BOX-PCR, Tavuk, Klonal heterojenite, *Escherichia coli*, (GTG)₅-PCR, Moleküler tiplendirme

INTRODUCTION

Escherichia coli is a major member of the human and animal normal gut microflora. Although commensal *E. coli* strains are nonpathogen, pathogenic types of *E. coli*,

including Enterotoxigenic (ETEC), Enterohemorrhagic (EHEC), Enteroaggregative (EAEC), Enteroinvasive (EIEC), and Enteropathogenic (EPEC) can cause intestinal diseases ^[1].

The ability to differentiate *E. coli* strains is critical for molecular typing, identifying bacteria at the strain level,



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studying bacterial population dynamics, and epidemiological surveillance of bacterial contamination; thus, it is necessary to apply rapid, reliable, and high-throughput typing methods [2]. Different phenotypic and biochemical characteristics have been previously used for the epidemiological investigations of *E. coli* [3]. However, the limitations of the phenotypically based typing methods (time consuming and lacking sufficient resolution amongst related strains), have led to the development of many DNA-based techniques. Therefore, a reliable genetic discriminatory method should be applied [4].

There are numerous methods to identify and characterize the diversity of bacteria, including; DNA banding pattern-based methods which classify bacteria according to the size of fragments generated by enzymatic digestion of genomic or plasmid DNA, DNA banding pattern-based methods which classify bacteria according to the size of fragments generated by PCR amplification, hybridization-based method, sequencing methods, detection of presence or absence of particular genes, and high Resolution Melting analysis [2]. All of the methods mentioned above are efficient typing methods because of revealing acceptable discriminatory power and reproducibility [5]. However, rep-PCR fingerprinting introduced by Versalovic *et al.* [6], is easy to set up, to use, to interpret, and inexpensive. Rep-PCR is a genotypic fingerprinting method that generates specific patterns by the amplification of repetitive elements present within bacterial genome [6]. Five rep-PCR methods are commonly used for genotyping of different bacterial strains including REP-PCR, ERIC-PCR, ERIC2 PCR, BOX-PCR and (GTG)₅-PCR among which BOX-PCR and (GTG)₅-PCR are of great interest [6]. Applying these methods will lead to the selective amplification of distinct genomic regions located between BOX and (GTG)₅ elements to produce specific banding profiles [7]. The use of BOX-PCR and (GTG)₅-PCR methods in the study of bacterial diversity has unveiled new insights in the composition of *E. coli* microbial communities and the number of data proving a considerable genomic diversity among *E. coli* strains is increasing steadily [8].

The ability to analyze *E. coli* populations by different methods not only can improve our understanding of the transport, viability and structure of *E. coli* populations but also can help us to develop strategies to identify bacterial pollution sources [9]. Thus, we conducted the current study to analyze clonal heterogeneity of *E. coli* isolated from chicken as well as to evaluate discriminatory power of BOX and (GTG)₅-PCR.

MATERIAL and METHODS

Ethics

For animal fecal samples, Permission was obtained from Alborz University of Veterinary Sciences, and Institutional Animal Care and Use Committee (IACUC) approved this

study. To collect samples, written information about the study was given to the husbandry owner and Informed consent was obtained.

Sampling, Bacterial Isolates, and DNA Extraction

This cross-sectional study was performed from August 1, 2015 to August 20, 2015. Applying sterile cotton-tipped applicators (swabs), 106 enteric specimens from healthy chickens (aging from 5-7 weeks) were obtained from private chicken husbandry Qadir in Karaj city (suburb of Alborz province with geographic coordinate of 35.8840059, 50.9716793). Since fecal samples were collected from living animal, no animal was sacrificed. For sampling, following autoclaving swabs in the capped tubes, they were inserted into the cloaca and rectum of chickens in such a manner as to insure the collection of fecal material. The swabs and adhering fecal material were then placed in the tube and quickly shipped to laboratory. To isolate *E. coli*, fecal swabs were inoculated into lauryl sulphate tryptose (LST) broth (Merck KGaA) followed by *E. coli* (EC) broth (Merck KGaA) and incubated at 44.5°C, and then the broth cultures were streaked on Eosin Methylene Blue Agar (EMB) agar (Merck KGaA). Colonies showing metal sheen were considered as presumptive *E. coli* isolates and were subjected to IMViC, tryptophanase and Beta-glucuronidase (Merck KGaA) tests for final confirmation [10]. Confirmed isolates were inoculated into sterile cryotube vials containing nutrient broth and were incubated overnight at 37°C. Sterile glycerol (Merck KGaA) was then added to each vial at a final concentration of 15% (vol/vol), and the vials were stored at -70°C. Following finishing sampling procedure, all frozen bacterial strains were revived in Brain Heart Infusion (BHI) broth under optimal growth condition and genomic DNA was extracted from the bacterial pellet applying AccuPrep® Genomic DNA Extraction Kit (Bioneer, South Korea). DNA was quantified and assessed for purity by spectrophotometry at 260 nm and 280 nm using a DR3900 Benchtop VIS Spectrophotometer (HACH, USA).

Molecular Fingerprinting

The primers used in this study were 5'-CTACGGCAA GGCGACGCTGACG-3' (Bioneer, South Korea) for BOX-PCR and 5'-GTGGTGGTGGTGGTGG-3' (Bioneer, South Korea) for (GTG)₅-PCR. The final reaction mixture for both protocols, consisted of 12.5 µL, 2x CinnaGen PCR master kit containing Hotstart *Taq* DNA Polymerase (recombinant), PCR buffer, MgCl₂, dNTPs, in addition to 1 µL (approximately 100 ng) template DNA and 10 pmols of each primers sets to the final volume of 25 µL. Amplification was performed with Veriti® 96-Well Thermal Cycler (Applied Biosystems) as follows: Initial denaturation (94°C for 5 min), followed by 30 cycles of denaturation (94°C for 20 s), annealing (30 s at 52°C for both protocols), extension (72°C for 1 min); and a final extension (72°C for 10 min). PCR products were resolved by horizontal electrophoresis in 1.5% (wt/vol) agarose (Bioneer, South Korea) and 1x Tris-

borate-EDTA (Merck KGaA) buffer. PCR products were evaluated by electrophoresis in 1% agarose gel (Merck KGaA) containing SYBR green (Thermo Scientific, Ukraine) and visualized by a Gel DOC™ XR⁺ (BIORAD) and analyzed by Image Lab™ 4.0 software.

Reproducibility Analysis of Rep-PCR

For reproducibility, five isolates were subcultured on tryptic soy agar (Merck KGaA) for 15 successive days (one subculturing per day). DNA was extracted after days 5, 10, and 15 of subculturing and used as template DNA for both (GTG)₅-PCR and BOX-PCR methods (a total of 15 genomic DNA).

Computer-Assisted Image Analysis and Cluster Assignment

Gel images were normalized, and fingerprints were assigned to isolates, with Bio-Rad's Image Lab™ 4.0 software. The positions of fingerprints on gels were normalized using generuler 100 bp plus ladder (Thermo Scientific, Ukraine) as the external standard in the range of 100 bp to 3.000 bp. For cluster analyzing, the data were converted to a binary matrix, where the digits 1/0 represent the presence/absence of the corresponding DNA band. Using the PAUP software 4.0 beta windows, strains were assigned to different clusters by calculating the similarity coefficients with the curve-based Pearson similarity coefficient. Similarity trees were generated using the unweighted-pair group method using average linkages. Clusters were initially assigned using the PAUP software on the basis of 80% similarity.

For both protocols, the Shannon-Weaver index for the diversity of isolates was calculated using the formula $H = -\sum (N_i/n) \log (N_i/n)$, as described before [11].

RESULTS

A total of 106 *E. coli* isolates were obtained from chicken fecal samples. Genomic DNA was successfully extracted

from all isolates and analyzed spectrophotometrically, confirming the purity and quantity of the DNA extracted.

Molecular typing of *E. coli* isolates by (GTG)₅-PCR generated 26 different bands ranging in size from 555 bp to 3.3 kb, while no common band was observed in all isolates. The majority of the isolates revealed complex banding patterns, while the most prevalent band (present in 73 isolates) was approximately 2.9 kb in size, and the least prevalent (present in 8 isolates) was approximately 1.87 kb in size. Among 106 isolates, two isolates were untypeable (generating no bands), while 104 isolates generated 100 unique (singletons) and 2 duplicate (GTG)₅-profiles (Fig. 1). The dendrogram generated six clusters (G1-G6) for the 106 strains tested. G2 contained 40 isolates followed by G3 (28 isolates), G5 (19 isolates), G2 (14 isolates), G4 (3 isolates), and G6 (2 isolates) (Fig. 2).

With the BOX-PCR method, profiles of *E. coli* strains revealed 10 amplified bands ranging from 0.7 to 3.5 kb, with various intensities. The most prevalent band which was present in 53 isolates was a band of approximately 2.9 kb in size, while the least prevalent band present in 8 isolates was approximately 1.87 kb. No common band was observed in all isolates. Because of low number of bands, the isolates did not reveal complex banding patterns. Visual comparison of the BOX-PCR banding results of 106 *E. coli* isolates revealed 50 unique BOX-PCR profiles in addition to 22 repetitive profiles, while 12 isolates showed no band. Based on the bands and dendrogram generation, isolates were grouped into six clusters (B1-B6). B3 contained 50 isolates followed by B2 (24 isolates), B4 (18 isolates), B5 (9 isolates), B6 (4 isolates), and B1 (1 isolates) (Fig. 3).

The degree of diversity calculated for the 106 isolates, using the Shannon-Weaver index, was 4.665 for (GTG)₅-PCR and 0.281 for BOX-PCR.

Following reproducibility testing, all isolates (days 5, 10, and 15 of subculturing) gave repeatedly the same

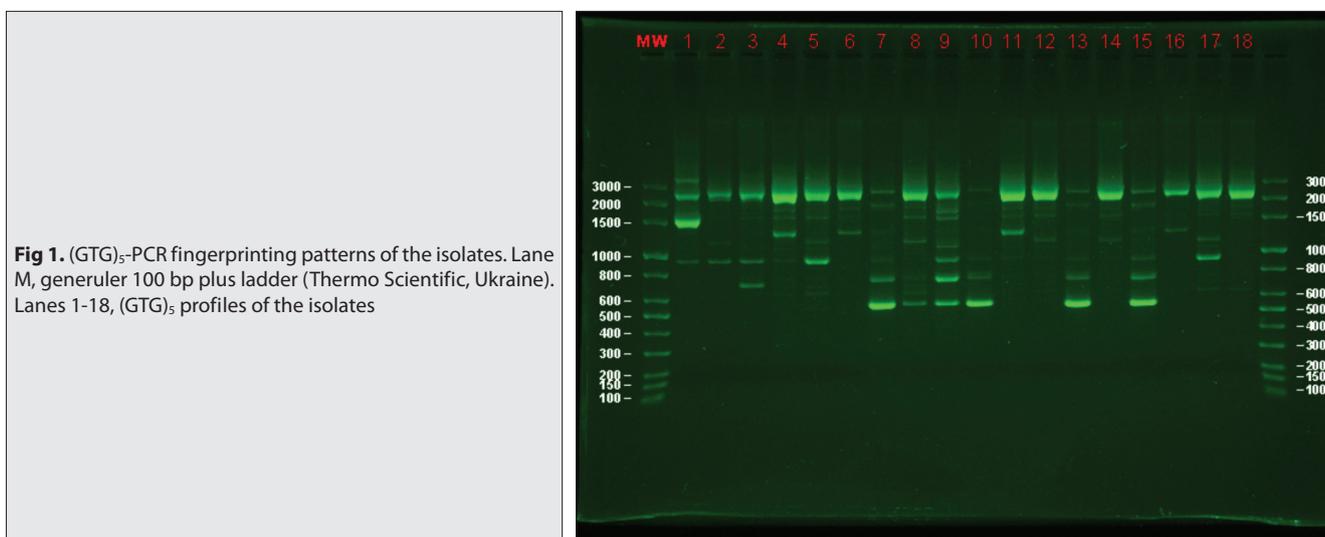


Fig 1. (GTG)₅-PCR fingerprinting patterns of the isolates. Lane M, generuler 100 bp plus ladder (Thermo Scientific, Ukraine). Lanes 1-18, (GTG)₅ profiles of the isolates

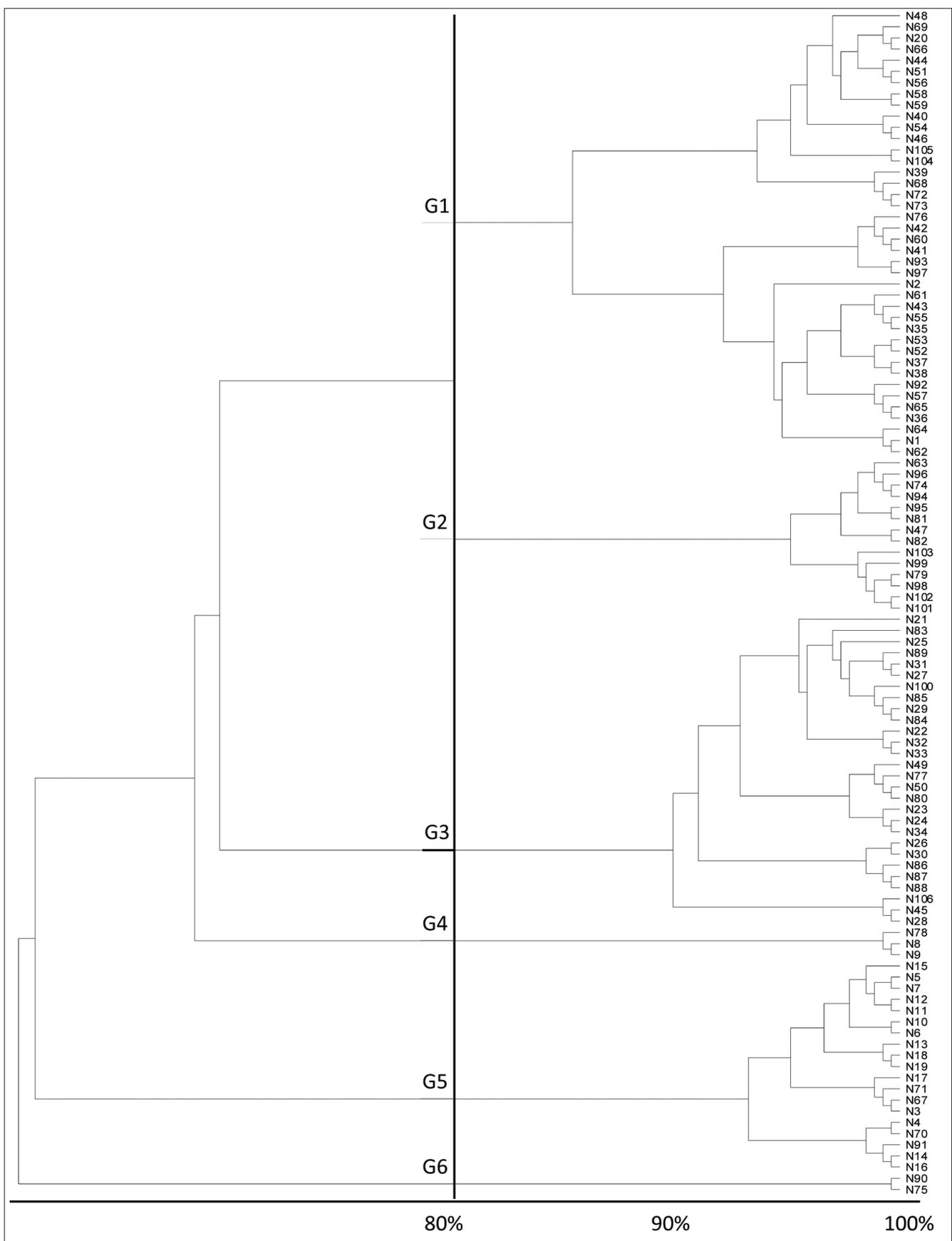


Fig 2. Dendrogram and cluster analysis of (GTG)₅-PCR fingerprints using the UPGMA clustering at a coefficient of 80% similarity. The bottom bar indicates the percent of similarity

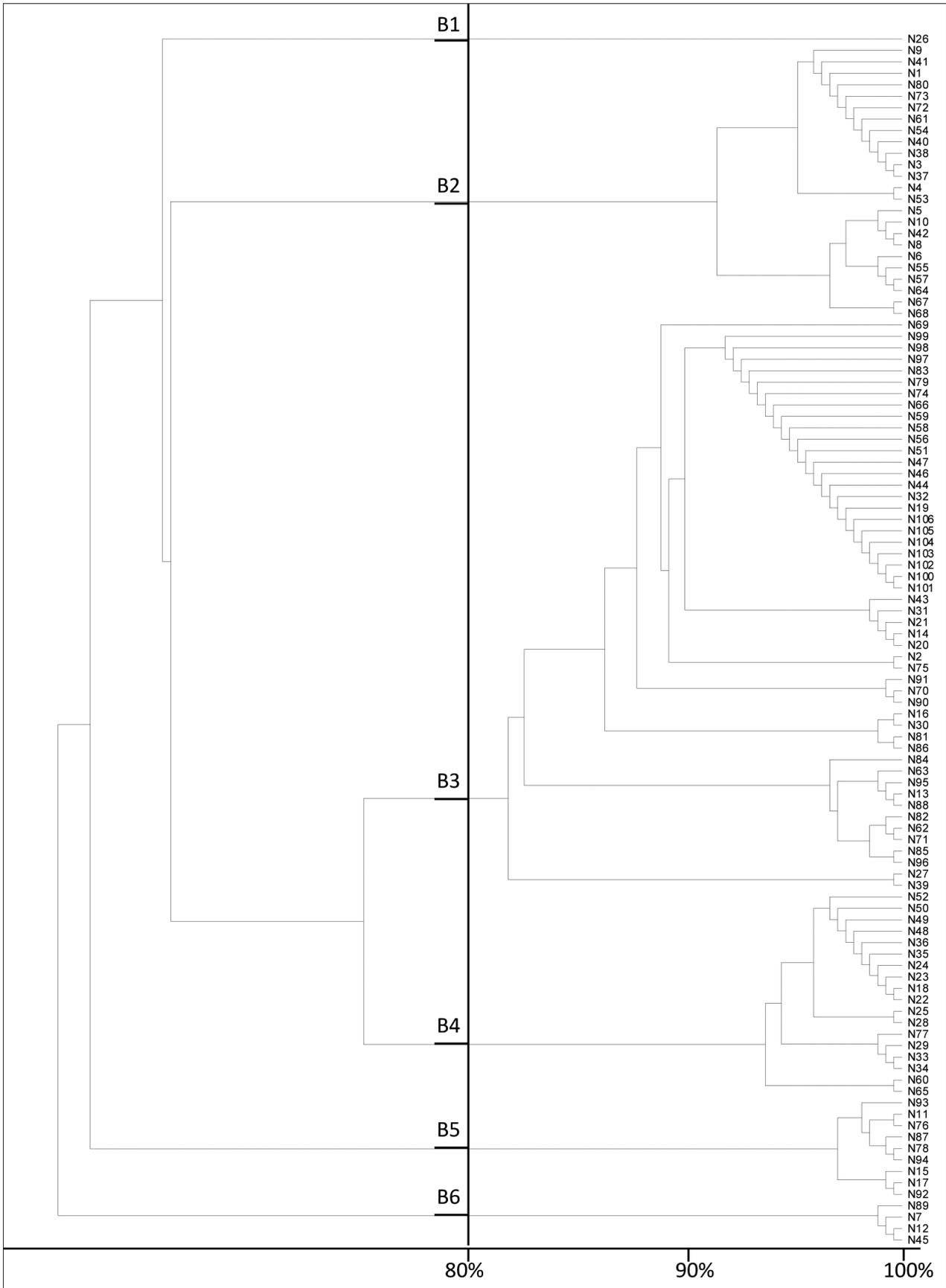


Fig 3. Dendrogram and cluster analysis of BOX-PCR fingerprints using the UPGMA clustering at a coefficient of 80% similarity. The bottom bar indicates the percent of similarity

band patterns without any difference due to missing or producing new bands. However, slight differences in intensity of some bands occurred.

DISCUSSION

Typing methods are efficient tools for the epidemiological study of bacteria. For a long time *E. coli* has been characterized by biotyping, phage typing, and serotyping with O-, H- and K- antigens [3]. From a biochemical and serological points of view, *E. coli* isolates have been demonstrated to be identical, but molecular studies have supported the existence of genetic variability among the isolates. Therefore, in recent years, traditional methods have been succeeded by molecular ones, among which pulse field gel electrophoresis is known to be the most efficient and gold standard of typing. PCR-based typing methods are other molecular tools which can be alternatives to PFGE, because they are fast and easy to setup and the results gained are to some extent comparable to PFGE [12]. However, the *E. coli* species has a comparatively clonal population structure, which can make the distinction of different isolates more difficult [13].

In this study we evaluated two PCR-based typing techniques, (GTG)₅ and BOX, which are frequently applied as molecular tools for fingerprinting of the different genus of the bacteria. The results obtained in this study revealed high clonal heterogeneity of the isolates. Furthermore, it was revealed that 98.5% of the chicken isolates were typeable by (GTG)₅-PCR, while just 53% of the isolates were typeable by BOX-PCR, this may confirm the robustness of (GTG)₅-PCR in comparison to BOX-PCR and consequently the lower applicability of BOX-PCR for typing compared with (GTG)₅-PCR. The lower Shannon-Weaver index with BOXA1 primer, the higher number of the untypeable isolates and the higher number of isolates with the same BOX fingerprints, would approve this claim. (GTG)₅-PCR is not only applicable for *E. coli*, but also is an efficient tool for fingerprinting other genus. For example, Pavel Svec *et al.* [14], described that (GTG)₅-PCR is a reliable and fast method for species identification of Enterococci because it was able to discriminate Enterococci strains from known and unknown sources.

To evaluate the discriminatory power of REP-PCR, ERIC-PCR, ERIC2-PCR, BOX-PCR and (GTG)₅-PCR for fingerprinting of *E. coli* isolates, Mohapatra *et al.* [15] conducted a survey which based on discriminant function analysis, they introduced (GTG)₅-PCR followed by BOX-PCR as the most robust molecular tools for differentiation of *E. coli* isolates, which confirms the higher accuracy of (GTG)₅-PCR in comparison to BOX-PCR.

Luc De Vuyst *et al.* [16], evaluated (GTG)₅-PCR with acetic acid bacteria and validated this technique with DNA:DNA hybridization data. They claimed that exclusive patterns

were obtained for most strains, suggesting that the technique can also be used for characterization below species level or typing of acetic acid bacteria strains.

In another survey, Mohapatra *et al.* [17] applied (GTG)₅-PCR to assess the ability of this typing method as a microbial source tracking tool. Following fingerprinting of 573 *E. coli* isolates from poultry and free living birds, Mohapatra *et al.* [17] indicated that (GTG)₅-PCR can be considered to be a complementary molecular tool for the fast determination of *E. coli* isolates identity and tracking the sources of fecal pollution.

Following (GTG)₅ fingerprinting of lactobacilli, Dirk Gevers *et al.* [18] found that (GTG)₅-PCR is a promising genotypic tool for fast and reliable speciation and typing of lactobacilli and other lactic acid bacteria. However, the result gained by Ma *et al.* [19] is not consistent with ours, because in a survey to differentiate between human, livestock, and poultry sources of fecal pollution, although the higher number of bands in (GTG)₅-PCR fingerprints could be observed, the discriminatory efficacy of BOX-PCR was superior to (GTG)₅-PCR. In another survey conducted by Dombek *et al.* [20] to differentiate *E. coli* isolates from human and animal sources of fecal pollution, BOX and REP primers were evaluated for DNA fingerprinting of *E. coli* strains. Using Jaccard similarity coefficients, Dombek *et al.* [20] managed to almost completely separate the human isolates from the nonhuman isolates.

To assess discriminatory power and suitability of BOX-PCR for bacterial source tracking, Carlos *et al.* [21], analyzed *E. coli* from different sources by BOX-PCR technique and a correct classification rate of 84% was achieved for strains from human and animal sources.

Based on our experiments, reproducibility results indicated that both (GTG)₅-PCR and BOX-PCR methods are of considerable repeatability and consistency. Following reproducibility testing, all isolates (days 5, 10, and 15 of subculturing) gave repeatedly the same band patterns without any difference due to missing or producing new bands. However, slight differences in intensity of some bands occurred. Identical results were gained by Pavel Svec *et al.* [14] and Gevers *et al.* [18] who tested reproducibility of the (GTG)₅-PCR fingerprinting for lactobacilli and Enterococcus. Although they occasionally found minor quantitative variations in band intensity, none of the strains tested in their study showed qualitative differences in the fingerprint patterns.

Similarly, a good reproducibility of rep-PCR fingerprinting was proved by Kang and Dunne [22]. They demonstrated high stability of fingerprints obtained from DNA isolated from 24, 48 and 72 h old bacterial cultures and from 5, 10 and 15 successive subcultured strains. Furthermore, Abby Yang *et al.* [23] reported that different concentration of template DNA, presence or absence of

bovine serum albumin, different annealing temperature and the growth phase of the culture template may not have notable effect on the BOX- fingerprints of *E. coli* of either gull or duck origins. However, Rasschaert *et al.*^[24] reported that reproducibility of (GTG)₅-PCR was poor between different PCR runs but high within the same PCR run. Of course it is noteworthy to indicate that for improving the reproducibility, we used Hotstart *Taq* DNA polymerase to eliminate any unspecific bands.

Regarding complex clonal heterogeneity of the isolates from chicken or any other sources, it is controversial to introduce the best molecular typing method, however, our results revealed that (GTG)₅-PCR method is more discriminative for typing of *E. coli* in comparison to BOX-PCR. Considering advantages and disadvantages of all typing methods, we can come to this conclusion that these approaches are complementary tools and combination of two or more different typing methods may lead to higher discrimination power rather than each of them when used individually.

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ETHICAL CONSIDERATIONS

Ethical issues (Including plagiarism, informed consent, misconduct, data fabrication and/or falsification, double publication and/or submission, redundancy, etc.) have been completely observed by the authors.

CONFLICTS OF INTEREST

The authors declare that they have no competing interests.

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