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African Journal of Bacteriology Research

Full Length Research Paper

Detection of *Mycobacterium bovis* in whey, by multiplex polymerase chain reaction (PCR) and bacteriological culture

Elaenia Minerva Gurrola-Mejía[2], Marco Antonio Santillán-Flores[1*], Laura Hernández-Andrade[1] and Marisela Leal-Hernández[1]

[1]Instituto Nacional de Investigaciones Forestales, Agrícolas y Pecuarias-CENID Microbiología. Carretera México -Toluca km. 15.5; Colonia Palo Alto, CP. 04110, Delegación Cuajimalpa, México.
[2]FMVZ-FES CUAUTITLAN UNAM, Carretera Cuautitlán-Teoloyucan Km 2.5, San Sebastián Xhala. Cuautitlán Izcalli, C. P. 54714, Estado de México.

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The objective of this study was to detect the presence of *Mycobacterium bovis* (*M. bovis*) in whey. A total of 233 cow milk samples were analyzed together with 26 tank milk samples that came from dairy herds of several states of the Mexican Republic (Querétaro, San Luis Potosí, Guanajuato, Hidalgo, Coahuila). DNA was obtained from whey and used for polymerase chain reaction-multiplex (PCR-M). Tuberculosis complex was first identified through the detection of gene RD1. Positive samples were subjected to a second PCR-M with the primers for gene RD9 to identify *M. bovis*. Samples were bacteriologically cultured using conventional techniques for the isolation of mycobacteria. Cohen's Kappa test (k) and Pearson's Chi^2 were carried out for statistical analysis. A 150 bp amplification product of the RD1 region was obtained, which corresponds to the tuberculosis complex, in 34/233 (14.59%) of the individual milk samples and in 4/26 (15.38%) of the tank milk samples. PCR-M with primer RD9, of the 34 individual samples and the 4 tank milk samples, gave an amplification product of 200 pb, which is the expected product for *M. bovis*. By bacteriological culture, six isolates were obtained; four in individual whey samples and two from tank milk samples, which were then classified by biochemical tests as *M. bovis*. The concordance between RD1, RD9 PCR-M and bacteriological culture was low, but there was a significant difference between diagnostic techniques with a P = 0.000. The results showed the potential of the PCR-M as a confirmatory test for the diagnosis of tuberculosis in cattle, as well as the advantage of using whey samples, that may be a possible source of infection for the herd and/or humans.

Key words: Bovine tuberculosis, whey, polymerase chain reaction-multiplex (PCR-M), RD1, RD9, milk, isolation.

INTRODUCTION

Mycobacterium bovis, is the etiological agent of bovine tuberculosis (TB), which infects a wide range of mammal species, including humans (Daza et al., 2017). In developing countries, TB is an important zoonosis, especially for high risk populations such as workers in dairy farms and slaughterhouses, veterinarians and

Table 1. Number of assessed individual and tank milk samples.

State of the republic	Dairy farms	Individual samples	Tank samples
Querétaro	2	30	2
Hidalgo	1	32	0
Guanajuato	20	92	22
San Luis Potosí	1	20	1
Coahuila	2	59	1

persons that consume fresh milk or cheese produced with non-pasteurized milk that comes from infected herds (Franco et al., 2013; Bapat et al., 2017). Tuberculosis in humans by *M. bovis* is less frequent in countries where the milk is pasteurized and bovine tuberculosis control and eradication programs are implemented. High prevalence in cattle facilitates airborne or digestive exposure to the bacilli, increasing the public health risk (Milián et al., 2012; Bapat et al., 2017). TB causes elevated economic loss to the cattle industry due to increased costs of control and eradication programs, as well as direct loss caused to the herd due to retention of carcasses in the slaughterhouse, reduction of 17 to 20% of milk production, 15% of calves' production and causing 20% premature discards (Boland et al., 2010; Iturra, 2016). Natural infection in bovines is direct or indirect, with respiratory and oral the main infection routes. *M. bovis* is mostly shed through expectoration and is considered that elimination through milk is less than 2% depending on the degree of infection the cow has and that the mammary lymph nodes and udder develop granulomatous lesions. *M. bovis* infected milk may contaminate milking equipment, floors, bedding, and containers used for retaining or storage of milk. Likewise, insufficient processing of milk may help the dissemination of the disease to other herds when the calves are fed with milk contaminated with *M. bovis* (Boland et al., 2010; Iturra, 2016). Confirmation diagnosis of TB is carried out by bacteriological culture of tissue samples with granulomatous lesions, nasal swabs and milk; although there is an inconvenience, the test takes four weeks for the development of the bacterial colonies and three weeks more for the typification by biochemical methods (Pérez et al., 2002; Clavijo et al., 2004; Michel et al., 2015).

Diagnostic techniques based in DNA amplification of *M. bovis* by polymerase chain reaction (PCR) have been amply described (Ramírez et al., 2004; Bapat et al., 2017). Diverse primers have been evaluated to amplify gene fragments that are specific of the *Mycobacterium tuberculosis* complex (MPB70, IS6110, IS1081) (Talbot et al., 1997; Diaz, 2013; Sweetline et al., 2017).

Comparative analysis between genomes allows the study of the evolution of a virulent strain to an attenuated variant. Even though *M. tuberculosis* as well as *M. bovis* and *M. bovis* BCG have a high degree of genome conservation, the presence of polymorphism regions which allow their differentiation has been detected (Diaz, 2013). Through of genomic hybridization studies, Mahairas et al. (1996), described that there were genetic differences or regions of differentiation (RD1 to RD16) in the *M. tuberculosis* genome, which allow to differentiate at the genetic level between species of the tuberculosis complex. Talbot et al. (1997), used primers for the RD1 region in a PCR-M test that allows the differentiation between pathogenic strains of the tuberculosis complex and the BCG strain. Parsons et al. (2002), assessed region RD9 to be used for PCR-M and conclude that this region allows for the differentiation between *M. bovis* and *M. tuberculosis*. Bovine tuberculosis is a disease that requires control and to have alternate diagnostic methods is desirable if they allow a more sensitive, specific, and quick diagnosis, when compared with the bacteriological culture. Likewise, if different types of samples can be used, such as bovine whey, it would facilitate the establishment of handling and control measures for the disease. The objective of this study was to detect the presence of *M. bovis* in bovine whey samples through a multiplex PCR and bacteriological culture.

MATERIALS AND METHODS

Sample collection

A convenience sampling was carried out with cooperating producers, in dairy herds with and without backgrounds of tuberculosis, as well as with or without mastitis problems, to determine the quality and safety of milk in relation to the shedding of *M. bovis*. A total of 259 milk samples were taken (233 individual samples from cows in different herds and 26 tank milk samples from different dairy herds in different states of the Mexican Republic) (Table 1). Each sample was approximately 50 ml of milk obtained prior to milking. The samples were collected in 50 ml falcon type sterile tubes and transported to the laboratory in an icebox with refrigerants. Milk samples were processed in the

*Corresponding author. E-mail: mayco1768@gmail.com or santillan.marco@inifap.gob.mx.

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following 24 to 48 h after they were obtained.

Obtaining whey

In a falcon tube, 10 ml of milk were placed and 50 µl of 1% chymosin solution was added. The samples were incubated in a double boiler at 37°C for 30 min, and centrifuged at 2500 rpm for 10 min. Whey was collected in 15 ml falcon type tubes and maintained in the freezer at -20°C until use. (Gurrola, 2017).

DNA extraction from whey

Two milliliters of whey were placed in microtubes and centrifuged at 12000 rpm for 15 min, supernatants were removed and to the pellet, 500 µl TE 1X and 100 µl of lysozyme (10 mg/ml) were added. The product was incubated at 37°C for 24 h; then, 75 µl of Proteinase K (10 mg/ml) and SDS (10%) were added, and incubated at 64°C for 10 min; a further 100 µl of NaCl 5M and CTAB (5 M)/NaCl (5 M) were added and incubated at 64°C for 10 min; finally, 750 µl of Chloroform-Isoamyl Alcohol (24:1) was added and centrifuged at 12000 rpm for 5 min. Aqueous phase was then transferred to a new 2 ml tube, 250 µl of Guanidine Isothiocyanate (5 M) and 250 µl Ammonium Acetate (7.5 M) were added and kept at 8°C, for 20 min; 500 µl of Chloroform-Isoamyl Alcohol (24:1) was added and centrifuged at 12000 rpm for 5 min. The aqueous phase was transferred to a 1.5 ml microtube and centrifuged at 12000 rpm for 5 min. The supernatant was eliminated and 500 µl of isopropyl alcohol was added to the pellet and incubated at -20°C overnight. This was centrifuged at 12000 rpm for 15 min, the supernatant was eliminated, and then washed with 70% ethanol for 5 min. The DNA pellet was allowed to dry at ambient temperature and hydrated with 100 µl of milli-Q water and stored at -20°C, until use in PCR-M (Ramírez et al., 2004).

Multiplex polymerase chain reaction RD1Test (PCR-M RD1)

The reaction was done using the following primers for the RD1 region: ET1 (5´-AAG-CGG-TTG-CCG-CCG-ACC-GAC-C-3´), ET2 (5´-CTG-GCT-ATA-TTC-CTG-GGC-CCG-G-3´) and ET3 (5´-GAG-GCG-ATC-TGG-CGG-TTT-GGG-G-3´), that amplify a 150 bp product of the tuberculosis complex, which allows differentiation between pathogenic strains of the tuberculosis complex from the BCG vaccine strains that amplify a 200 bp product (Talbot et al., 1997). For each sample, the following were used: 12.5 µl PCR Master-Mix (4 mM $MgCl_2$, 0.4 mM of each dATP, dGTP, dCTP, dUTP, 0.05 U DNA polymerase), 1 µl of each of the primers ET1 (5 pmol), ET2 (25 pmol), ET3 (5 pmol), 5 µl nuclease-free water, and 10 µl of DNA (6 ng/µl.) obtained from whey. *M. tuberculosis* H37Ra (ATCC # 25177) and *M. bovis* BCG (ATCC # 35734) DNA were included as positive controls. The thermocycler program was as follows: one cycle at 94°C/5 min, followed by 35 cycles at 94°C/40 s, 65°C/40 s, 72°C/40 s, and a final extension at 72°C/4 min, holding the reactions at 4°C until transferred out. The amplification products were visualized in 2% agar gel stained with ethidium bromide. The samples that came out positive to PCR-Multiplex RD1 were subjected to PCR-Multiplex RD9.

RD9 PCR-M

The following primers were used: RD9 FF (5´-GTG-TAG-GTC-AGC-CCC-ATC-C-3´), RD9 Int (5´-CAA-TGT-TTG-TTG-CGC-TGC-3´) and RD9 FR (5´-GCT-ACC-CTC-GAC-CAA-GTG-TT-3´) that amplify a 300 bp product of *M. tuberculosis* and a 200 bp product of *M. bovis* (Parsons et al., 2002). For each sample, the following were used: 12.5 µl PCR Master Mix; 1 µl of each of the primers RD9 FF (5 pmol), RD9 Int (25 pmol), RD9 FR (5 pmol); 5 µl of nuclease-free water and 10 µl DNA (6 ng/µl) obtained from whey. As positive controls, *M. tuberculosis* H37Ra (ATCC #25177) and *M. bovis* BCG (ATCC #35734) DNA were used. The same thermocycler program described earlier was used. The amplification products were visualized in 2% agar gel stained with ethidium bromide.

Bacteriological culture

The whey samples were cultured in duplicate with the technique described by Perez et al. (2002) using Lowenstein Jensen and StoneBrink culture media, and incubated at 37°C during nine weeks. Obtained bacterial growth was stained with Ziehl Neelsen and typified by biochemical methods (niacin test, catalase, tween 80 hydrolysis; nitrate reduction, pyrazinamidase) as well as pigmentation production and growth rate.

Statistical analysis

Statistical analysis was carried out using the STATA® 7.0 software package (StataCorp LP, College Station, TX, USA). Cohen's Kappa test (k) or inter-rater agreement index was used to establish the association between the results obtained with the RD1, RD9 PCR-M and culture. Differences between diagnostic techniques were tested using Pearson's Chi^2 (χ^2).

RESULTS

A 150 bp amplification product was obtained from the RD1 PCR-M tuberculosis complex in 34/233 (14.59%) individual milk samples (Figure 1 and Table 2) and 4/26 (15.38%) tank milk samples (Table 3).

In all cases of PCR-M amplification using RD9 primers of the 34 individual samples and 4 tank samples, a 200 bp band was obtained, which was the expected product for *M. bovis* strains (Figure 2 and Tables 2 and 3).

Six isolates were obtained by bacteriological culture, four in the individual whey samples and two in the tank samples. The isolates were cultured for four weeks and were positive to the Ziehl Neelsen stain, classifying them as Acid-Fast Bacillus (AFB). Using biochemical methods strains were classified as *M. bovis* (Tables 2 and 3).

The concordance between RD1, RD9 PCR-M and bacteriological culture was low, but there was a significant difference between diagnostic techniques with a P = 0.000 (Table 4).

DISCUSSION

M. bovis isolation percentage in this study was 1.7% in individual milk samples and 7.6% in tank milk samples. To have isolates positive to AFB depends on many factors, amongst them that the agent is being shed in the sample that is obtained, and its viability and adaptability; even if the percentage of isolates that were obtained in the study may be considered of low importance, it

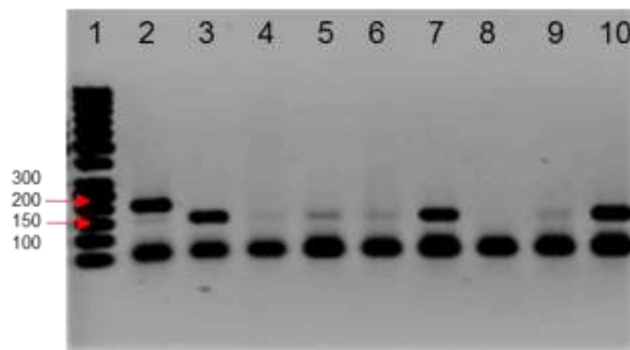

Figure 1. PCR-M RD1. Lane 1: MW (50 bp), 2) *M. bovis* BCG (200 bp), 3) *M. tuberculosis* H37*Ra* (150 bp) 4-7, 9, 10) amplification products (150 bp) obtained from DNA bovine whey samples (200 pb), 8) DNA negative simple.

Table 2. Positive results by State, identified by PCR Multiplex, using RD1 and RD9 primers on individual whey DNA samples (P=0.000).

State	Individual samples	PCR RD1	PCR RD9	*M. bovis* culture
Querétaro	30	0	0	0
Hidalgo	32	0	0	0
Guanajuato	92	18	18	4
San Luis Potosí	20	4	4	0
Coahuila	59	12	12	0

Table 3. Positive results by State, identified by PCR Multiplex, using RD1 and RD9 primers on whey DNA of tank samples (P=0.000).

State	Tank samples	PCR RD1	PCR RD9	*M. bovis* culture
Querétaro	2	0	0	0
Hidalgo	0	0	0	0
Guanajuato	22	4	4	2
San Luis Potosí	1	0	0	0
Coahuila	1	0	0	0

confirms the shedding of viable AFB that can be recovered in whey which could be the origin of infections in animals and humans (Boland et al., 2010; Michel et al., 2015; Bapat et al., 2017). The probability of obtaining positive cultures from bovine milk samples is generally low; less than 2% of the cows that have tuberculosis shed the bacillus by milk. This occurs when the animal has a generalized infection and open lymph nodes, as well as involvement of the udder and the mammary lymph nodes. Conversely, the elimination of mycobacteria by aerosols and nasal discharge occurs intermittently (Clavijo et al., 2004).

A study carried out in Brazil (Franco et al., 2013) detected, respectively 7 and 9% presence of *Mycobacterium* species, in individual and tank milk samples, identifying other mycobacteria (*Mycobacterium fortuitum*, *Mycobacterium flavescens*, *Mycobacterium smegmatis*, *Mycobacterium vaccae*) besides *M. bovis*, as possible milk contaminants. Bacteriological culture results of this study may be considered low in comparison to those reported by Franco et al. (2013), yet the difference in all cases was the identification of *M. bovis* and the lack of sample contamination with environmental mycobacteria.

The presence of pathogenic microorganisms in food and food-borne illnesses represent essential and growing public health problems due to the increase in frequency, the surging of new transmission forms, growth of vulnerable population groups and the social and economic impact they have (Yánez et al., 2008; Bapat et

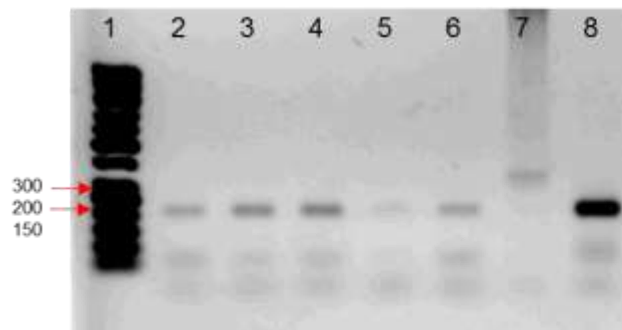

Figure 2. PCR-M RD9. Lane 1: MW (50 bp), 2-6) amplification products (200 bp) obtained from bovine whey samples (200 pb), 7) *M. tuberculosis* H37Ra (300 bp), 8) *M. bovis* BCG (200 bp).

Table 4. Cohen's Kappa (K), Standar Error (SE), Z value (ZV), and χ^2, comparing RD1, RD9 PCR-M and Culture.

Parameter	K	SE	*ZV*	χ^2
PCR-M RD1 vs. Culture	0.2432	0.0401	6.07	0.000
PCR-M RD9 vs. Culture	0.2431	0.0401	6.07	0.001
PCR-M RD1 vs. RD9	1.0	0.0613	16.31	0.000

al., 2017). Whey is a byproduct of the cheese industry and represents 80 to 90% of the total processed milk volume. It contains 40 to 80% protein concentrates, which allow an ample use of these byproducts, mainly in the food industry, such as for the substitution of other ingredients and components in drinks, yogurt, spreadable cheeses, curd, sausages, bakery, confectionery, and even in the pharmaceutical industry. It can be used as a food source for pigs and bovines, and therefore it is considered necessary that it receives special treatment to eliminate a possible load of pathogenic agents to avoid them becoming a potential source of infection for humans, as well as animals.

Doran et al. (2009), described a case of bovine tuberculosis in Ireland in which a family of six were affected due to the consumption of non-pasteurized milk and derivatives. In the farm, all adult bovines, as well as 80% of the calves that were fed from a milk storage tank, tested positive to the tuberculin test, later presented tuberculous lesions, and *M. bovis* was isolated from udder and milk. There are regions in Mexico where more than 28% of milk or cheeses produced with non-pasteurized milk are consumed raw, and whey is used as feed supplement for ruminants and pigs and therefore there is a high risk of contamination with *M. bovis* (Gurrola, 2017). It is important to underline that in Mexico there are not many well documented studies of bovine tuberculosis in humans that detect the origin in consumption of non-pasteurized milk. Yet the danger is latent due to the consumption of non-pasteurized milk habits. Therefore, control measures must be taken for milk products, in relation to possible infectious agents that could be contaminating the product, as well as the containers that are used for it.

Biotechnology advances have allowed the development of alternative diagnostic methods that provide advantages in relation to efficiency, sensitivity, and reduction of detection time (Ramírez et al., 2004; Sweetline et al., 2017). These methods are quick because they are based in the determination of nucleic acids and have the property of being specific.

PCR needs the correct selection of the target sequence to be identified in the microorganism genome of interest. In this study, PCR-M was carried out to diagnose bovine tuberculosis with a sensitivity of 95% and a specificity of 100% (Talbot et al., 1997; Ramírez et al., 2004; Das et al., 2007), based on the identification of two Difference Regions, 1 and 9 (RD) of the tuberculosis complex. In this study, 257 bovine cattle milk samples were analyzed using PCR-M of RD1 and RD9, of which the presence of *M. bovis* DNA was detected in 14.8% of them. The use of the genetic markers of regions RD1 and RD9, in PCR-M for the diagnosis of bovine tuberculosis in DNA samples obtained from whey has allowed the establishment of a sensitive and specific technique to detect the presence of *M. bovis* DNA that takes less time than the bacteriological culture, which is considered the confirmation proof for the diagnosis of bovine tuberculosis, but it has the

inconvenience that the time for the growth of mycobacterial colonies is slow and its sensitivity is below 50% (Sweetline et al., 2017).

The use of conventional techniques for bacteriological culture and PCR in milk samples are key tools to determine milk quality in relation to the presence of *M. bovis*, since it allows the detection of animals shedding the bacterium in milk (Sweetline et al., 2017). A control program should be implemented to eliminate cows with tuberculous mastitis, including the appropriate training of dairymen and population in general about the risks when consuming raw milk and its derivatives, when they come from tuberculosis infected herds. With this, the infection risk for humans and replacement calves should be reduced.

Conclusion

The results in this study show the potential that the use of PCR-M with RD1 and RD9 primers has as a diagnostic method for bovine tuberculosis, as well as the advantage of using whey samples that may be a possible source of infection for herds and/or humans.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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Multidrug resistant *Escherichia coli* isolated from asymptomatic school going children in Kibera slum, Kenya

Gitahi N. J.[1*], Gathura P. B.[1], Gicheru M. M.[2], Githinji T. W.[4] and Nordin A.[3]

[1]Department of Public Health Pharmacology and Toxicology, Faculty of Veterinary Medicine, University of Nairobi, Kenya.
[2]Department of Zoological Sciences, School of Pure and Applied Sciences, Kenyatta University, Nairobi, Kenya.
[3]Department of Energy and Technology, Faculty of Natural Resources and Agricultural Sciences, Swedish University of Agricultural Sciences, Sweden.
[4]School of Biological and Physical Sciences, University of Nairobi, Kenya.

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Pathogenic *Escherichia coli* are of different types, currently grouped into six groups depending on the virulence gene(s) they possess. This study isolated pathogenic *E. coli* from 580 stool samples obtained in the month of August, 2016. The samples were obtained from asymptomatic school going children in one of the biggest urban slums in Kenya. Ten primary schools were randomly sampled and 40 to 80 stool samples collected from each school depending on the school population. Both gender and age were considered when sampling. Data obtained was analysed using single factor ANOVA to test association between school location and levels of infection with pathogenic bacteria. A total of 244 (17%) samples had *E. coli*. Out of these, 38 (6.5%) were shown to have one or a combination of the pathogenic genes, namely: *ipaH, virF, st2, daaE, eae, aafII, stx1, bfp, lt* and *stII* and were thus classified into seven groups. Of the pathogenic isolates 35 (21.2%) were multidrug resistant. There was an association between school location and the prevalence of pathogenic bacteria. In conclusion, asymptomatic school going children in the slum were found to be infected with multidrug resistant pathogenic *E. coli*.

Key words: Enteropathogenic, *E. coli,* multidrug resistance, school going children, asymptomatic.

INTRODUCTION

Escherichia coli form the normal flora of human and other mammals as the gut microbiota (Kaper et al., 2004; Fratamico et al., 2016). *E. coli* colonizes the gastrointestinal tract of infants within hours of birth, establishing mutual benefits with the host (Kaper et al., 2004). On colonization, *E. coli* will remain harmless to the host, only causing infection when the mutual environment is disturbed and in immune suppressed hosts. Pathogenic *E. coli* are of different types depending on the virulence gene(s) they possess (Kaper et al., 2004).

*Corresponding author. E-mail:nduhiugitahi@gmail.com. Tel: 254-0722339553.

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Differentiating pathogenic strains has largely depended on serotyping methods (Fratamico et al., 2016).

Though serotypes are useful in pathogenic *E. coli* identification, they cannot be used to characterize the clinical syndromes of the strains. It is also a time consuming and expensive method, requiring well trained manpower. Identification of the specific virulence factors(s) in the pathogenic bacterial has been achieved through genotypic methods that detect specific genes encoding the pathogenic characteristics of the *E. coli* (Kaper et al., 2004). Based on the presence of virulence genes and ability to cause disease pathogenic *E. coli,* were classified as following pathotypes: Enteropathogenic *E. coli* (EPEC), enterotoxigenic *E. coli* (ETEC), enteroinvasive *E. coli* (EIEC), enteroaggregative *E. coli* (EAEC) shiga toxin– producing *E. coli* (STEC) and diffusely adherent *E. coli* (DAEC). Hybrid pathotypes have also been described including the enteroaggregative hemorrhagic *E. coli* (EAHEC) carrying both the STEC and EAEC associated genes (Fratamico et al., 2016), such hybrid was reported by Franz et al. (2011) as EAHEC serotype 0104:H4.

E. coli are one of the bacterial pathogens of international concern regarding antibacterial resistance according to WHO (2014). *E. coli* can develop resistance through different ways but mostly through mutation, resulting in fluoroquinolone resistance. The bacteria can also acquire mobile genetic elements including; plasmids, transposons, integrons and gene cassettes resulting in resistance to penicillins (ampicillin or amoxillin) as well as to third generation cephalosporins (WHO, 2014). Resistance to cephalosporins has been shown to be caused by extended spectrum beta-lactamases (ESBLs), enzymes which destroy beta–lactam antibiotics. *E. coli* has the ability to acquire ESBLs, causing them to be resistance to Beta-lactams (Downie et al., 2013). The presence of diarrheagenic *E. coli* in asymptomatic individuals has been reported in several studies and it is attributed to a number of observations including, development of protective antibodies early in life against diarrheagenic *E. coli,* as well as host susceptibility. This has been shown in children growing up in endemic areas (Quiroga et al., 2000). Though the actual process leading to diarrheal is not clearly understood (Donnenberg and Finlay, 2013), non-specific host barriers, such as internal microbiota, intact mucus layer and epithelial cell layers have been postulated to prevent diarrheal episodes (Levine and Robin, 2012). Bacterial factors inclining some pathogens to diarrhoea has also been shown in EPEC as a factor (Hu and Torres, 2015). Research clearance was from National Commission for Science, Technology and Innovation (NACOSTI) and Ethical clearance through the Kenyatta University Ethical Review Committees. The objective of this study was to determine the prevalence of pathogenic *E. coli* infection in asymptomatic school going children and establish their antibiotic resistance patterns using phenotypic and genotypic methods.

MATERIALS AND METHODS

Study area

The study was carried out in primary schools located in Kibera informal settlement, Nairobi County, Kenya. Kibera is located at an altitude of 1670 m above sea level, at longitude 1° 17° East and latitude 36°, 50° South and it is about 140 Km South of Equator. Due to poor infrastructure and drainage the slum is heavily polluted by human and animal excreta, organic and inorganic waste littering the open sewage lines. The area can be characterised by lack of sufficient sanitation and poor water supply which results in frequent waterborne disease outbreaks (Feikin et al., 2011).

Escherichia coli culture

Five grams of faecal sample was weighed and suspended in 45 ml of buffered peptone water to make a 1:9 (10^{-1}) dilution. Further, a 10-fold serial dilutions were made; 10^{-2}, 10^{-3}, 10^{-4} in sterile physiological saline solution (0.85%). Chromocult coliform agar (Merck Millipore Corporation, Germany) was prepared according to the manufacturer's instructions and kept at 50°C in water bath prior to use. One millilitre of the diluted and vortexed samples (10^{-3}, 10^{-4}) was transferred into well labelled sterile petri dishes and 15 ml of the cooled medium was added. The plates were thoroughly mixed avoiding spillage. The media was allowed to solidify at room temperature before being incubated at 44.5°C, 18 h. Characteristic colonies were examined, counted and recorded as total thermo-tolerant coliforms (appearing as salmon red) or total *E. coli* (appearing as dark-blue to violet colonies). *E. coli* were confirmed by overlaying the dark-blue to violet colonies with Kovacs reagent and observing for a cherry-red colour change. Four characteristic colonies of *E. coli* per stool sample were sub-cultured on to clean Chromocult agar to obtain distinct colonies. Colonies embedded in the media were sub-cultured by piercing the pour plate agar using a sterile straight wire. The pure cultures as well as a pool of all the four different isolates from a single sample were stored in skimmed milk at -80°C for further characterization.

DNA preparation from bacteria colonies and multiplex polymerase chain reaction (PCR)

Only pure bacterial cultures were used for DNA preparation. Three distinct colonies were picked from agar plates containing pure culture with a sterile wire loop and suspended in 0.5 ml sterile distilled water. The suspension was then placed in a water bath and heated to boil for 30 min. After cooling to room temperature the suspension was centrifuged at 2000 g and the supernatant was decanted and stored at -20°C as DNA templates.

Due to the large number of *E. coli* isolates, first the pools were sub-cultured, DNA isolated and tested for the presence of pathogenic *E. coli* using multiplex PCR. For pools that were positive, individual isolates making that specific pool were individually analysed. The PCR was carried out using a Veriti 96 wells thermocycler (Applied Biosystems, model 9902, Singapore) in 0.2 ml PCR tubes. A 25 µl reaction mix was prepared by mixing 12.5 µl of PCR master-mix (Taq polymerase 0.05 U/µl, 0.4 mM of each dNTP, 4mM $MgCl_2$) (Biolabs Inc., New England) with ten pairs of specific primers (10 pmol) at 0.5 µl each (Vidal et al., 2005) and 2.5 µl DNA template. Primers used for amplification of products encoding for pathogenic genes in *E. coli* are shown in Table 1.

E. coli were amplified with a program of initial heating at 94°C for

Table 1. Primers used for patho-typing *E. coli* isolates.

Primer sequence 5'-3'	PCR product size (pb)	Encoded gene	References
F-CTC GGC ACG TTT TAA TAG TCT GG R-GTG GAG AGC TGA AGT TTC TCT GC	933	*ipaH*	Vidal et al. 2005
F-AGC TCA GGC AAT GAA ACT TTG AC R-TGG GCT TGA TAT TCC GAT AAG TC	618	*virF*	Vidal et al. 2005
F-ATC CTA TTC CCG GGA GTT TAC G R-GCG TCA TCG TAT ACA CAG GAG C	584	*stx2*	Berlutti et al. 1998
F-GAA CGT TGG TTA ATG TGG GGT AA R-TAT TCA CCG GTC GGT TAT CAG T	542	*daaE*	Vidal et al. 2005
F-TCA ATG CAG TTC CGT TAT CAG TT R-GTA AAG TCC GTT ACC CCA ACC TG	482	*eae*	Stacy-Phipps et al. 1995
F-CAC AGG CAA CTG AAA TAA GTC TGG R-ATT CCC ATG ATG TCA AGC ACT TC	378	*aafII*	Vidal et al. 2005
F- CAG TTA ATG TGG TGG CGA AGG R- CAC GCA ACA ATG TAA CCG CTG	348	*stx1*	Berlutti et al. 1998
F-GGA AGT CAA ATT CAT GGG GGT AT R- GGA ATC AGA CGC AGA CTG GTA GT	300	*bfp*	Stacy-Phipps et al. 1995
R-TCC TTC ATC CTT TCA ATG GCT TT F-GCA CAC GGA GCT CCT CAG TC	218	*lt*	Stacy-Phipps et al. 1995
R-AAA GGA GAG CTT CGT CAC ATT TT F-AAT GTC CGT GCG TTA GGA C	129	*stII*	Stacy-Phipps et al. 1995

5 min followed by 94°C, 1.5 min minutes denaturation, 1.5 min at 60°C primer annealing and at 72°C for 1.5 min elongation for 35 cycles, with a final extension of 72°C for 7 min. The PCR products were kept at -20°C until gel electrophoresis was done. The PCR products were visualized following electrophoresis in a 1.5% agarose (Genetics analysis grade, Fisher Scientific, New Jersey) gel stained with 0.02% ethidium bromide and amplicons identified against molecular marker (50 bp DNA ladder, England Biolab) run long side the samples. A UV transilluminator digital camera (Gelmax 125 imager, Cambridge UK) with a UVP software interphase computer (Upland CA, USA) was used to visualize DNA bands relative to the molecular weight maker. For confirmation the positively identified PCR products were submitted for sequencing (ABI 3500XL Genetic Analyzer).

Antimicrobial sensitivity test (AST) for confirmed *E. coli* isolates.

Bacteria inoculum for the antimicrobial resistance test was prepared by touching the top of at least 2 to 3 well isolated colonies with a sterile wire loop and then transferred into sterile normal saline solution (0.85% NaCl). The inoculum was emulsified on the inside of the tube to avoid clumping of the cells and turbidity adjusted to a 0.5 McFarland. A standard bacterium, *E. coli* ATCC 25922 was included in every test as antibiotic sensitivity breakpoint control. Six antimicrobial discs were placed on a single Muller Hinton agar plate with a sterile multidisc dispenser. One hundred and sixty five (165) *E. coli* isolates were tested against twelve antimicrobial agents namely; Tetracycline (30 mg, TE); Ciprofloxacin (5 mg, CIP); 30 mg Naladixic acid (NA); 25 mg Trimethoprim-Sulfamethaxone (SXT); 30 mg Ceftriaxone (CRO); 10 mg Amoxycillin-clucianic acid (AMC); 30 mg Ceflazime (CAS); 10 mg Ampicillin (AMP); 30 mg Chloramphenicol (C); 10 mg Gentamycin (CN); 10 mg Streptomycin (S), CXM; 30 mg Cefuroxicin (CXM) according to the CLSI (2012) guidelines. The 12 antimicrobial agents are classified into 9 subclasses by CLSI (2012).

Genotypic characterization of bacteria isolates for antimicrobial resistance

Seventy five of the 165 *E.coli* isolates tested for antimicrobial sensitivity by the disc diffusion test were selected for genotypic characterization based on their phenotypic profiles. The isolates were tested for the presence of genes encoding for resistance to tetracyclines, quinolones and sulfonamides. Presence of four tetracycline resistance genes; *tet A, tet B, tet C* and *tet O* and two sulfonamides resistance genes namely *sul1* and *sul2* was tested. Multiplex PCR was carried out and primers used for amplification of products encoding for the resistant genes to the three antibiotic classes (Table 2).

RESULTS

A total of 221 stool samples had *E. coli* isolated, out of which 38 (6.5%) were pathogenic *E. coli*. These were distributed at different frequencies in the 11 schools studied with 4 (36%) schools having none isolated (Table 3). Using multiplex PCR, 83/555 (15%) pooled *E. coli* positive samples were shown to have one or a combination of the virulence genes and were thus classified into seven groups. Seventeen (3.1%) of the pools had the *eae* gene and were classified as atypical enteropathogenic. Thirty two (5.8%) pooled isolates were

Table 2. Primers used for identifying tetracyclines and sulphonamides encoding genes in selected bacteria isolates.

Primer sequence 5’-3’	PCR product (pb)	Encoded gene	References
F-GTGAAACCCAACATACCCC R-GAAGGCAAGCAGGATGTAG	577	*Tet A*	Randall et al., 2004
F-CCTCAGCTTCTCAACGCGTG R-GCACCTTGCTGAGACTCTT	635	*Tet B*	Randall et al., 2004
F-ACTTGGAGCCACTATCGAC R-CTACAATCCATGCCAACCC	880	*Tet C*	Van et al., 2008
R-AACTTAGGCATTCTGGCTCAC R-TCCCACTGTTCCATATCGTCA	515	*Tet O*	Abdi-Hachesoo et al., 2014
F-TTCGGCATTCTGAATCTCAC R-ATGATCTAACCCTCGGTCTC	822	*Sul1*	Van et al., 2008
F-TTCGGCATTCTGAATCTCAC R-ATGATCTAACCCTCGGTCTC	285	*Sul2*	Van et al., 2008

Table 3. Prevalence of pathogenic *E. coli* per school in the study area.

Schools	No sample per school	*E. coli* (Isolated) per school	Pathogenic *E. coli* n (%)
A	59	42	6 (14.2)
B	38	2	2 (0)
C	62	37	3 (5.4)
D	44	0	0 (0)
E	79	42	10 (23.8)
F	19	15	3 (20)
G	80	49	14 (28.6)
H	53	1	1 (0)
I	30	0	0 (0)
J	69	30	1 (0.33)
K	47	3	1 (33.3)
Totals	580	221	38
% Prevalence in the study area			6.5%

enterohemorrhagic forming the highest number followed by enterotoxigenic at (13/555) 2.3%, diffusely adherent at (12/555) 2.16%, enteroaggregative at (4/555) 0.72%, typical enteropathogenic at (3/555) 0.54% and lastly enteroinvasive at (2/555) 0.36%. A hundred and twenty three (20%) single *E. coli* isolates from 113 faecal samples were subjected to PCR and characterized into 6 pathotypes, with EHEC having 33 (27%) isolates, EPEC (typical and atypical) with 30 (24%) isolates, DAEC at 28 (23%) isolates, ETEC at 26 (21%) isolates, EAggEC with 5 isolates and lastly EIEC had only one isolate (Table 4). Multiple infections with more than one pathogenic *E. coli* were observed in 10 (8.8%) individuals. Three (2.0%) children had EHEC and EAggEC combined infection, another 4 (3.5%) children had EPEC and EIEC. A combined infection of EHEC, EAggEC and DAEC was observed in one (0.9%) child. Also observed in single individual child were EHEC together with EIEC and EPEC with EAgg EC (Table 4).

Multidrug resistant profiles of *E. coli* isolates

One (0.6%) *E. coli* isolate was resistant to the eight antibiotics tested, 3 (1.8%) were resistant to seven drugs, 6 (3.6%) were resistant to six drugs, 18 (10.9%) were resistant to 5 drugs while 33 (20%) were resistant to 4 drugs. Thirty five (21.2%) were shown to be resistant to 3 drugs (Figure 1).

Phenotypically the antimicrobial subclass with the highest number of resistant isolates was sulfonamides with 115 (70%) isolates, this was followed by aminopenicillin with 97 (59%) isolates, tetracyclines had 91 (55%), aminoglycoside with 74 (45%) isolates, fluoroquinolones at 37 (22%) and phenicols had 24 (15%) resistant isolates. The other three subclasses had less than 10 (6%) resistant isolates (Figure 2).

Genes encoding for resistance to three antimicrobial subclasses were tested in 74 out of 165 phenotypically resistant isolates. One (1.4%) isolate had *TetA* gene, 6

Table 4. Molecular characterization of pooled positive *E. coli* from stool (N=555).

PCR genotype	Prevalence (n)	Genes demonstrated
Enterohemorrhagic *E. coli* (EHEC)	5.76%, (32/555)	*stx1,stx2, eae*
Atypical Enteropathogenic *E. coli* (EPEC)	3.06%, (17/555)	*eae*
Typical Enteropathogenic *E.coli* (EPEC)	0.54%, (3/555)	*eae, bfp*
Enterotoxigenic *E.coli* (ETEC)	2.34%, (13/555)	*lt, stII*
Diffuse adherent *E.coli* (DAEC)	2.16%, (12/555)	*daaE*
Enteroaggregative *E.coli* (EAggEC)	0.72%, (4/555)	*aafII*
Enteroinvasive *E.coli* (EIEC)	0.36%, (2/555)	*IpaH, VirF*

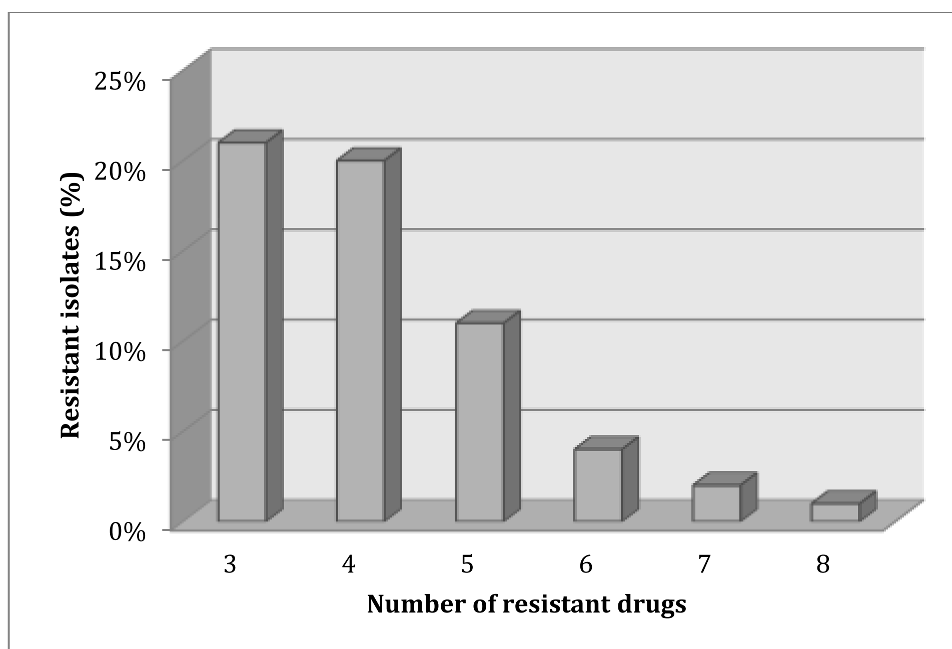

Figure 1. Percentage of multidrug resistant *E. coli*.

(8.1%) had *TetB* gene, 14 (19%) had *TetC* gene and none had *TetO* gene. Sulfonamides gene (*Sul1*) was only found in 10 (14%) isolates while none of the isolates had the *Sul2* gene.

Molecular sequencing of selected *E. coli* isolates

The *eae* gene encoding the outer membrane protein was demonstrated in 5 *E. coli* isolates submitted for gene sequencing. The *eae* gene in the 5 isolates had blastn alignment identity of 99%, 98 and 94% with standard *E. coli* O157:H7, EP 057 isolate and *E.coli* O157:H7, respectively. These similarities confirmed the identity of the PCR products obtained from *E. coli* isolated from the asymptomatic school going pupils. Heat labile enterotoxin (*Lt)* genes were demonstrated as eltB and eltA flanked by IS600 and IS1294. Two isolates processing the heat labile enterotoxin B encoding genes were submitted for sequencing. The sequences alignment with blastn gave a 99 and 84% identity for isolates 103a and 582a, respectively. Heat stable enterotoxin (*StII* gene) was shown in two isolates which were submitted for sequencing. The PCR products for the *StII* gene were sequenced and the sequences aligned using blastn giving 74 and 100% alignment respectively. One isolate with the *bfpA* gene encoding for major structure subunit of bundle forming pilus, had gene alignment identity at 99%.

DISCUSSION

Pathogenic *E. coli* were characterized using multiplex PCR to demonstrate the presence of characteristic virulence genes. A prevalence of 17% obtained in this study was much lower compared to Rono et al. (2014), who reported a prevalence of 34.2% in diarrheal

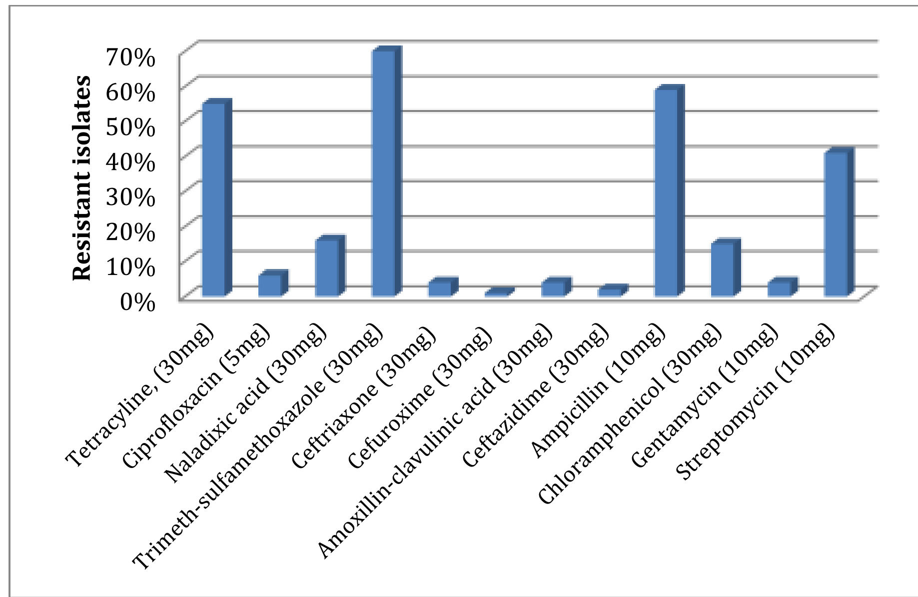

Figure 2. Drug resistant patterns of pathogenic *E. coli* isolates from stool samples, N=165.

samples. The difference in the observations being attributed to the fact that while this study sampled asymptomatic pupils, Rono et al. (2014) worked with diarrheal patients.

Molecular analysis of pathogenic *E.coli* isolates revealed that 13 (2.3%) samples had the enterotoxigenic (ETEC) genes, *LT* or the *ST 2*. Vidal et al. (2005) described ETEC having one or more enterotoxins; heat labile (LT) *LT1* and *LT2* or the heat stable (ST) as *STa* and STb. In the present study, three children had typical EPEC with both the *eae* and *bfp* genes. Another 17 pupils had atypical EPEC having only the *eae* gene. These findings compares well with those reported by Bugarel et al. (2011) and Vidal et al. (2005) who observed a ratio of 1:3 and 1:4, typical to atypical EPEC respectively. EPEC has proteins involved in attaching and effacement of the host cells microvilli. It has the EPEC adherence factor (EAF) plasmid and gene cluster encoding the bundle–forming pili (*bfp*) gene. Typical EPEC are those isolates with the EAF plasmid, those with the *bfp* gene but lack the EAF plasmid are classified as atypical EPEC. EIEC had the least number of isolates with only 2 (0.36%) children positive. This compares with other findings where none out of 509 stool samples yielded EIEC (Vidal et al., 2005). Rono et al. (2014) made comparable observations, with EIEC isolation being the second last frequently isolated, at 3 out of 100 samples. EIEC possess a gene located in a virulence plasmid (Plnv) 140 MDa encoding type III secretion system. Highest number of isolates were found to be EHEC, with 32 out of 555 children positive. The isolates had a combination of *eae* gene with either *stx1* or *stx2* genes. Several authors have reported different distributions in the prevalence of different pathogenic *E. coli*. Some have reported EAEC as the most frequent; others had EAggEC being the most frequent, while ETEC was shown by a different study as the most frequent. EPEC has also been identified by a different study as the most prevalent in Kenya (Sang et al., 2012a; Sang et al., 2012b; Makobe et al., 2012; Bii et al., 2005).

Unlike Vidal et al. (2005) who observed one patient out 509 with mixed enteropathogenic *E. coli*, this study had 10 individuals out of 555 with mixed enteropathogenic infections. While 9 of the 10 had combinations of two different enteropathogenic *E. coli*, one of the children had three pathogenic *E.coli* characterized (EHEC, EAggEC and DAEC). These findings are an indication that clinical symptoms alone, may not be conclusive in the diagnosis of entropathogenic *E. coli* infections. Resistance to sulfonamides was the highest at 70%, this was followed by ampicillin at 59%, tetracycline 55%, streptomycin 41%, nalidixic acid at 16% and chloramphenicol at 15%. The findings in this study agree with Christabel et al. (2012) who reported high resistance of environmental isolates from Kibera slums. Though the frequencies were not in agreement with this study, the general profile of resistance is comparable. In this study prevalence of resistance to cephalosporins (ceftriaxone, cefuroxime and ceftazidime) were very low at 4, 0.7 and 2%, respectively, resistance by *E. coli* to cephalosporins is mainly by the extended spectrum beta-lactamases (ESBLs) enzymes. These enzymes are able to destroy most of the beta-lactam antibiotics (WHO, 2014). ESBLs can be transferred between bacteria species. In a report on resistance to antibacterial drugs in selected bacteria of international concern WHO, (2014) the report gave a

range of resistance *E. coli* to third generation cephalosporin in thirteen African Countries at 2-70%. This was within the levels of this study which had 4% resistant to ceftriaxone.

Only 31 (18.7%) pathogenic isolates were sensitive to all the 12 antibiotics tested, 21 (12.7%) were resistant to only one of the 12 antibiotics, while the other 113 (68.5%) were resistant to more than 2 antibiotics. Isolates resistant to 3 or more antibiotics were 35 (21.2%) thus classified as multidrug resistant (MDR) isolates. Those resistant to any four of the 12 antibiotics were 33 (20%), those resistant to 5 antibiotics were 18 (10.9%), resistant to 6 antibiotics were 6 (3.6%) and those resistant to 7 antibiotics were 3 (1.8%) isolates. Multidrug resistant *E. coli* has been reported on environmental isolates at 40% from the same study area. These findings have slightly lower frequency compared to those reported in this study, which could be attribute to the differences between human isolates and environmental isolates. The findings however agrees with Kipkorir et al. (2016) who reported 42.2 % MDR *E. coli* isolates from faecal specimens obtained from patients ages 2 weeks to 82 years. The patients had gastroenteritis and the study was in Kitale, Kenya. Oundo et al. (2008) also reported 65.5% MDR *E. coli* isolates from asymptomatic food handlers in Kenya, which was very close to this study's 68.5%. They however reported resistance to cefuroxime at 6.9% which was much higher than 0.7% in the current study, which could be attributed to difference in the age of sampled study subjects.

Sequenced *TetA* gene from enteropathgenic tetracycline resistant *E. coli* showed 99% homology with related *TetA* sequences in GenBank. The fact that 55% of tested *E. coli* were resistant to tetracycline and the demonstration of *TetA* genes in some of the isolates would suggest that genes were freely transferable between bacteria *in-vivo* in cases of multi-bacterial infections observed in this study. The homology observed is a frequent finding with tetracycline resistance encoding genes in *E. coli*. *TetA* gene has been reported by Abdi-Hachesoo et al. (2014) to be located on bacterial mobile elements and thus horizontally transferable among bacteria strains. The 99 and 100% homology obtained after sequences alignment of the virulence genes confirmed identity and virulence of the pathogenic *E. coli* isolates. This proved that diarrheagenic *E. coli* were indeed isolated from asymptomatic children in the current study. Similar findings have been reported by Quiroga et al. (2000), who demonstrated the presence of EPEC, DAEC, ETEC and EAgEC in symptomatic infants. The authors also described 7.5 months of life as the earliest time of *E. coli* colonization.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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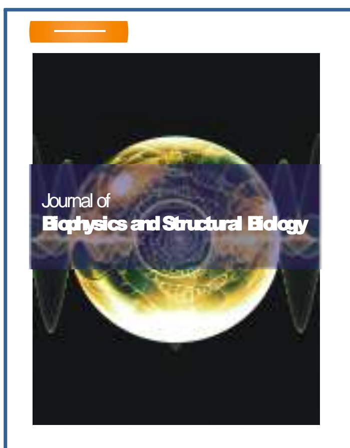

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