Diversity of Escherichia coli isolates from milk obtained along the Nairobi dairy value chain by DNA fingerprint analysis

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Abstract

Background

The Nairobi dairy food system is highly complex and involves informal and formal dairy production and marketing channels. The sector comprises numerous small-scale interlinked actors characterized by diverse food handling practices that may affect product safety. This cross-sectional study sought to analyze the diversity of *Escherichia coli,* which contaminates cow milk, in material obtained from the supply chain serving Nairobi's rapidly urbanizing city in Kenya. The GTG_5 fingerprinting method was used to determine the diversity of 107 *E. coli* isolates obtained from milk. Dendrograms were used to display *E. coli* genetic diversity patterns within and between farms from different types of samples. These included raw, pasteurized, processed fermented, and home-made fermented milk sourced from various nodes, including farms, milk bars, milk vending machines, roadside milk vendors and shops.

Results

Analysis of the first dendrogram with 46 *E. coli* isolates recovered from various milk types from different nodes showed three major clusters based on bacterial banding patterns. A large proportion of the subsequent sub-clusters in these phylogenies revealed a similarity matrix of between 50 – 70 % among isolates from the same location. Dendrograms derived from analysis of *E. coli* at farm level showed that most isolates from milk samples obtained from the same farms did not cluster together which strongly suggests variation in the recovered *E. coli* strains and in the populations of *E. coli* in those farms. These findings indicate distinct bacterial milk contamination sources and not as a result of the clonal spread of certain strains.

Conclusions

These results show that the source of milk contamination is diverse and occurs at several points along the value chain. Therefore, policy on the management of food safety (including control of milk-borne diseases) should not only focus on activities at few nodes but along the entire value chain to ensure milk safety.

Highlights

- A similarity matrix of between 50 – 70 observed in clusters of isolates from the same location
- There was diversity among *Escherichia coli* strains recovered from milk within the same farms
- Milk contamination possibly through distinct sources rather than as a result of the clonal spread of particular strains

Introduction

Milk consumption is popular across the globe due to its nutritional benefit and provision of proteins, calcium, vitamin B12, iodine and magnesium (1). In addition, demand for consumption of milk and milk products is expected to triple in sub-Saharan Africa, by 2050, influenced by the rise in population, urbanization and increased preference for animal source foods (2). The FAO predicts that demand for milk in Kenya will increase by 175%, rising gradually from 4,839,000 tons (2010) to 7,513,000 tons (2030) and 13,298,000 tons in 2050 (3). However, this predicted demand will be unmatched with production (2). It follows that numerous value chains may evolve to support milk supply into the country and therefore understanding the structures and functionality of the milk systems is critical in addressing targeted food safety interventions (4–7).

Food systems present complicated networks, especially in urban areas where production and distribution is via often complex value chains (6, 8–10). Such system complexities are excellent avenues for introducing and transmitting pathogens, including food hazards among other food safety risks (11–13). Due to high nutritional content, milk is an ideal medium for the growth of bacterial contaminants (14), among other food safety hazards (15–17). The World Health Organization (WHO) estimates that 31 of the 32 diseases reported globally between 2007 and 2015, were caused by foodborne hazards (18).

Microbial food contamination is one of the leading concerns in food systems in developing countries, mainly due to inadequate and poorly designed food safety structures and policies (19, 20). Studies conducted in Kenya show that milk may be contaminated with bacteria, including those of zoonotic importance at different value chain levels. For example, the presence of bacteria like *Mycobacterium bovis* in cattle may represent a potential risk to humans infection through consumption of unpasteurized milk (21, 22). In addition, some studies have identified *Brucella abortus* and *E. coli* O157:H7 in marketed milk which indicates a weakness in disease management at farms (23–25). Furthermore, the high prevalence of brucellosis in humans has previously been linked to raw milk consumption (26–28).

The processes involved in the introduction and transmission of diseases (or food safety hazards) in humans, animals and the environment are complex (29). This is mainly due to the numerous interlinkages of the food networks and linkages involved from production through processing, marketing and disposal of waste products, with each step providing opportunities for risks (8). A guideline developed by FAO on animal disease risk management amplifies the need to thoroughly understand the livestock value chains in the context of operations and stakeholders' decision-making to enhance effective and targeted interventions (30).

Among the many bacterial contaminants in raw and processed milk, *E. coli* is the most common (31). *E. coli* is a gram-negative rod-shaped, facultative anaerobic, coliform bacterium of the genus *Escherichia* and is commonly found in the lower intestines of warm-blooded animals where they live as harmless normal gut microbiota (32, 33). However, some serotypes can cause serious food poisoning in their hosts and occasionally are responsible for food contamination events that have prompted product recalls (34). The boiling of raw milk is known to kill most bacterial contaminants, but the heat-resistant toxins such as enterotoxins can still be harmful to the final consumers (35).

About 50% of *E. coli* population reside in secondary habitats like soil, plant surfaces, ground water, and different environments, allowing them to colonize new hosts (36, 37). Here, they can replicate to establish distinct stable strains different from the original host-adapted populations (38–40). Contaminated soil is particularly significant in promoting environmental selection pressure, which enriches the locally adapted genotypes that may contribute to the genomic diversity, potentially transmitting stress tolerant strains to new hosts through food or water (41). The high degree of genome plasticity results from gene losses and gains through horizontal transfer (42, 43). The resultant heterogeneity in these organisms makes it possible for *E. coli* to reside in many environments (44) and therefore, the organism can serve as a marker for microbial movement (45–47). This phenomenon is essential in understanding critical points of disease emergence (or food safety) and transmission as well as understanding the linkages between the sources of pathogen and the route of their transmission (39, 48, 49).

In Nairobi, milk is marketed through a complex framework that is comprised of small-scale actors, usually perceived to work independently, but previous research shows that these actors are highly connected and interdependent (4, 5). Milk value chains from different sources intersect and the introduction of food safety hazards may happen at any point of the value chain. However, there is insufficient information to demonstrate such points, which may serve as critical points for designing and implementing any interventions. Repeated sequences in bacterial genomes such as Enterobacterial Repetitive Consensus Sequences (ERIC) and the GTGGTGGTGGTGGTG (GTG_5) can be helpful in conducting such analysis, particularly to investigate the clonal variability of the bacterial isolates (50). Although these techniques have a low resolution as compared to whole-genome sequencing and work best in related bacterial species, the methods can be exploited to assess genetic diversity in a group of similar bacterial species (51). This can help track the flow of micro-organisms at a broad level. According to FAO, an understanding of the correct source of contaminants and avenues for transmission is critical in maintaining the integrity of food systems, including executing better management practices to prevent the spread and emergence of diseases (30).

The current study's main objective was to analyze the *E coli* isolated from milk obtained from different nodes of the Nairobi dairy value chain to understand the diversity in those bacteria populations as a marker for the milk system's stability. This approach presents a tactical analytical method that would inform policy makers' strategic interventions in complex food value chains. We believe that the findings in Nairobi will be informative for broader urban African populations.

Materials And Methods

Selection of study units

This study was part of a suite of projects aiming to understand the emergence of disease in urban populations. Elsewhere (Kiambi *et al* - in progress), the methods for data collection and sampling for analysis of total coliforms (TCC) from which the current study draws its *E. coli* isolates have been documented. In brief, 290 cow milk samples were collected from 63 farms, five milk collection centers, 37 kiosks, 17 milk bars, 14 roadside vendors, three restaurants, two milk vending milk machines, two mobile traders and one supermarket. The types of samples obtained included raw milk (N=203), home-made fermented milk (N=12), home-made yogurt (N=3), pasteurized milk (N=35), Ultra Heat Treated milk (N=13), processed yogurt (N=13) and processed, fermented milk (N=11). The samples were collected from two geographical locations, Uthiru (Dagoretti and Kabete sub-locations) and Kasarani. Uthiru location is a peri-urban area in Nairobi and commercial dairy farming is a common practice. On the other hand, Korogocho is an informal settlement (slum) and livestock keeping is not a major activity, although some people keep a few dairy cows, pigs, poultry, sheep and goats (52).

Milk sampling commenced early in the mornings and ended by 10 am. The farmer was requested to milk about 50mls directly into a sterile barcoded falcon tube. Still, if the farmer was unable to milk, the household was requested to give whatever remained from the last milking (even if it was pooled). Participants were requested to transfer milk directly into the sterile barcoded falcon tubes to obtain about 50mls of milk from the other nodes (retail and bulking centers). However, if the milk was in packets or sealed bottles, the entire content was purchased. All milk samples were immediately placed in a cool box packed with ice packs and transported to the University of Nairobi (UoN), Public Health, Pharmacology, and Toxicology laboratory within 2-4 hours of collection. At the laboratory, various tests, including enumeration of total coliforms and isolation of *E. coli* were carried out.

Isolation of *Escherichia coli*

For each milk sample, pre-enrichment was done in 0.1% sterile peptone water by incubating a 10-fold dilution of the sample for 24 hours at 37^0C. A primary culture was then obtained by transferring about 5µL (loopful) of each dilution using a sterile wire loop to MacConkey Oxoid™ agar and incubation was done at 37°C for 24 hours to obtain distinct colonies. This was followed by a purification process that entailed sub-culturing four pink, dry and pinpoint (rounded colonies) on MacConkey Oxoid™ agar and incubating at 37°C for 24 hours (secondary culture). A single characteristic colony from each sub-culture plate was emulsified into an Eppendorf containing a 0.5ml sterile normal saline for running various biochemical tests. Biochemical tests included the Indole test, Methyl red test, Voges-Proskauer test and Citrate utilization test (IMVIC) as described elsewhere (53). Biochemical identification of *E. coli* was based on the IMVIC results as follows: Indole positive (+), methyl red positive (+), vogues Proscar negative (-) and utilization of citrate negative (-). Pure *E. coli* isolates from the secondary purification were then cultured onto nutrient agar Oxoid™ at 37^0C. The isolates were then stocked in sterile skimmed milk and gradually frozen at -20^0C to -40^0C and finally -80^0C for future analysis.

Selection of isolates for fingerprinting

The typing work was conducted at the Centre for Microbiology Research laboratories, Kenya Medical Research Institute (KEMRI), Nairobi. Two hundred and sixty-eight (268) *E. coli* isolates were successfully revived from the stocked 290 cultures. The first set of analyses that was input into the Gelcompar®2 software comprised 46 isolates (clarity of the dendrograms reduced with inputting very many isolates) that were randomly selected to represent the bacterial diversity in the three study areas. This was followed by a purposeful selection of isolates from farms to represent bacterial diversity patterns at farm level. These included all 13 isolates from Kasarani farms, 21 from Dagoretti and 27 isolates from Kabete farms.

Reviving of *E. coli* isolates and extraction of DNA

The stored colonies were revived by picking a loopful of each frozen stored isolate and sub-culturing at 37^0C for 24 hours in Eosin Methylene Blue agar (EMBA). The culture media was prepared according to the manufacturer's instructions and stored at 40^0C until use. The colonies with the characteristic green metallic sheen growth were selected using a sterile wire loop and streaked on Muller Hinton agar (MHA) and incubated 24 hours at 37^0C. Some colonies were then harvested for Deoxyribonucleic Acid (DNA) extraction and the remainder stored in skimmed milk at -80^0C for future use.

The boiling method of DNA extraction was used in this study (54). Revived pure *E. coli* colonies on MHA plates were emulsified in 1mL distilled DNase/RNase-free water and boiling done at 95°C for 15 minutes to achieve bacterial cell lyses. Separation of bacterial nucleic material was done by centrifuging the boiled content at 14000 revolutions per minute for 5 minutes. The supernatant containing the extracted DNA was transferred into a sterile Eppendorf tube and stored at -20°C.

Fingerprinting

The polymerase chain reaction (PCR) method was used to amplify the target repetitive extragenic palindrome sequence present in bacterial DNA (51). The GTG_5 (5'-GTG GTG GTG GTG GTG-3') single primer was used in the PCR amplification of target DNA (55). According to the manufacturer's instructions, the reactions were done using PuReTaq (Ready-To Go PCR beads - GE Healthcare, Bukinghamsire UK). Thermo-cycling steps included: initial denaturation at 95°C for two minutes, annealing at 40°C for one minute, a short extension step at 65°C for eight minutes and then a final extension at 65°C for eight minutes were followed. Staining of the DNA loaded in agarose gel was done using SYBR™ green stain. Gel electrophoresis was performed at 80 Volts in 1× Tris-acetate EDTA (TAE) buffer for one hour to separate the amplified genomic fragments.

Data analysis and interpretation of dendrogram

Fingerprint analysis was done using the Gelcompar®2 software version 6.6 BioNumerics softwareavailableonlineat (https://download.appliedmaths.com/sites/default/files/download/bn_quickguide_0.pdf). Digital images were entered into Gelcompar®2 software and edited to greyscale for ease of bands definition. Analysis of the banding patterns to generated a dendrogram was done based on the Pearson coefficient using the unweighted pair group arithmetic mean (UPGMA) method (56). Bacterial diversity was evaluated by analyzing isolates clustering patterns based on sample type, farm of orgin and similarity index. Isolates in cluusters that had a similarity of $\geq$ 80% were considered to be closely related. In comparison, those with less than 80% were deemed to be diverse (51).

Results

The dendrogram generated from the subsample of 46 *E. coli* isolates derived three main groups of the bacteria designated as G.1, G.2 and G.3 (**Figure 1)**. Further, the three groups subdivided into six clusters, designated as A, B, C, D, E, F and G for ease of analysis and distinction. None of the bacterial clusters and consecutive sub-clusters had 100% similarity as presented in the generated dendrogram (**Figure 1)**. A large proportion of bacterial isolates from the same locality clustered together, with cluster B dominated by bacterial isolates from Kabete location while cluster D, F, G had bacterial isolates predominantly from milk samples obtained from Kasarani. However, the two clusters A and C contained isolates of *E. coli* from milk sampled from Dagoretti location, indicating a potential common origin or source of contamination. Cluster E had the most diverse isolates recovered from milk samples obtained from Kabete, Kasarani and Dagoretti locations. Except for a single sub-cluster consisting of 2 isolates from the same farm in Kasarani location, there was no indication of clustering based on similar farms, milk bars, milk vending machines, roadside milk vendors or one sold at the shops. In summary, the diversity in most clusters as presented in this dendrogram suggests a broad diversity of *E. coli* amongst the bacterial populations contaminating milk, and a lack of a common source across all the milk between and within locations. This suggests multiple sources of milk contamination at different points in the chain, such that interventions have to target multiple sources.

A similar lack of isolate relatedness was observed in a dendrogram generated from 13 isolates from milk sampled from one area alone, Kasarani. Isolates obtained from raw milk samples within the same farm did not cluster together therefore signaling that the farm itself is not a determinant of similarity and emphasizing the diversity of *E. coli* contamination in milk even at small geographical scales **(Figure 2)**.

A dendrogram for isolates recovered from Kabete location revealed four (4) major cluster groups with similarities of $\geq$50% **(Figure 3)**. A similarity of more than 90 % among isolates from different farms was noted in **Group 1 and 3**. Although the reason for these tight clustering was not established, there is a high possibility that there is common source of these *E. coli* milk contaminants within Kabete location**.** A possibility of common source contamination was further strengthened by observations that noted close clustering of a few isolates recovered from the same farm (Farm 39 and 40 in Group 1). However, further analysis indicated that most isolates within the same farm in this location were diverse, as inferred by separate *E. coli* isolates within the same farm (**Figure 3)**.

Similar observations were noted among *E.coli* isolates in the Dagoretti location that showed close clustering with a similarity matrix of $\geq$80% among few isolates within the same farm (Farm-04 in Group 1 and 2 in **Figure 4**) Also comparable to Kasarani and Kabete, most isolates within the same farm in Dagoretti location were diverse and clustered into separate groups.

Discussion

The emergence and transmission of foodborne pathogens is dynamic and complex (57), and associated with several drivers resulting from, amongst others, urbanization and adaptation of micro-organisms (57–59). These factors are usually propagated by the complex system linkages associated with social,

ecological, environmental dynamics and economic factors (29, 60). Historically, approaches dealing with disease threats are mainly reactive, meaning that activities transpire during or after an outbreak (58). However, contemporary research suggests that more comprehensive approaches of combating health threats would involve a forecasting approach (61, 62), or prediction of broad patterns in pathogen evolution or defining the underlying causes of emergence (30, 63). For this purpose, molecular techniques have proven to be valuable tools in informing decision-making during outbreak investigations and response (64, 65), identification of the source of infection (65–68) as well as detecting emergence of new pathogens (67, 69, 70). Such benefits can be achieved by applying affordable and available low-resolution fingerprinting methods that can help determine the diversity and possible sources of microbial contaminants in the milk distribution chain. Low-resolution typing has its limitations but as used here, is useful in distinguishing diversity at a broad level.

Cluster analysis using ERIC PCR typing done in this study showed that *E. coli* populations isolated from raw, pasteurized, fermented milk and yogurt were diverse. *E. coli* contamination in the system studied does not appear to derive from a common source linked to value chain nodes; farms, milk collection centers, milk bars, restaurants, roadside vendors, shops/kiosks, supermarkets and milk vending machines. We detected a wide range of genetic diversity, as determined by the methods employed, implying several distinct contamination sources along the value chain. At the level of an individual farm within the study, *E. coli* isolates from milk samples were also genetically diverse, with no consistent grouping detectable in the cluster analysis, indicating a wide range of contamination sources even at that scale. Husbandry practices in the peri-urban farming systems such as those studied may not prioritize hygiene for the animals or the products they produce, and the generalist nature of *E. coli* thus results in milk being contaminated from a variety of sources during different stages of production, storage, transportation and processing (44).

The presence of *E. coli* in raw milk is a common finding and may be associated with the health status of the cows (71–74), which may directly contaminate the harvested milk (75, 76). Other possible sources of contamination of milk with *E. coli* observed in this study include the environment of the cow due to poor and unhygienic milk handling techniques like milking with unclean hands, dirty milking equipment and unwashed cow udders at milking (71, 74, 77) as well as prolonged storage of raw milk at ambient temperature (78, 79), in dirty containers. On the other hand, the presence of *E. coli* in pasteurized milk, as detected in our samples, may indicate inadequate pasteurization processes or contamination of the product post pasteurization (80). This latter finding is particularly worrisome from a public health point of view, indicating inadequacies in the process of pasteurization in the informal supply systems we studied.

Milk distribution in Kenya is mainly unregulated, with more than 80% of the milk being sold by small-scale informal traders (81). Being ‘informal’ means that the enterprises are not registered or licensed to operate and as such are very difficult to regulate (82, 83). Cognizant of this, there have been attempts by the Government of Kenya to organize the sector through training and certification of informal traders (84, 85). However, a subsequent study found that numerous challenges and governance issues hamper compliance with formal rules geared towards the promotion of food safety in the dairy system (5). While Kiambi (2020b) pointed towards the need for policymakers to address governance challenges, our current study provides further evidence that milk acquires contamination at multiple points of the milk value chain, emphasising the need to critically analyse the system prior to designing and implementing any interventions. This is further underscored by the fact that the Nairobi dairy system is comprised of multiple value chain actors who are mainly small-scale operators and are highly interlinked and interdependent (4). Nairobi's situation is likely to similar in other developing African cities (25, 86, 87).

A major limitation in interpreting results from this study is that the sample size was limited to milk samples collected within one city and within only a few locations within it. Therefore, although the dairy sector is crucial in Kenya, and while our results are likely to point to similar issues elsewhere in the value chain, the results cannot be generalized to the entire country. However, this analysis is critical in that it suggests domains for broader assessment and highlights the value of low-resolution molecular typing tools that are affordable in this setting for routine use, in better understanding the diversity of contamination sources in a complex food value chain. Further research is required to understand the social, ecological, environmental and economic determinants of the propagation of foodborne contamination in the milk system, and more detailed genomic studies of *E. coli* are indicated to better understand the biology of that organism specifically.

Conclusion

Our analyses indicate that *E. coli* isolated from milk at a range of value chain nodes (farms, milk collection centres, milk bars, restaurants, roadside vendors, shops/kiosks supermarkets and milk vending machines) did not cluster when a low resolution genetic typing tool (ERIC-PCR) was used to detect diversity. This implies that milk contamination happens at multiple points along the value chain and the sources of this contamination are diverse and unlikely to represent a single source. Policy on control and prevention of milk-borne disease and infections should focus on activities at the farm and the entire value chain. Deliberate measures should be put in place to ensure milk safety by enforcing hygiene measures and improved milk handling from production to the consumer.

Abbreviations

CGIAR - Consultative Group on International Agricultural Research

DNA - Deoxyribonucleic Acid

E. coli - Escherichia coli

EMBA - Eosin Methylene Blue agar

ESEI - Environmental & Social Ecology of Human Infectious Diseases Initiative

FAO - Food and Agriculture Organization

GTG5 - 5'-GTG GTG GTG GTG GTG-3'

ILRI - International Livestock Research Institute

IMViC - Indole, Methyl red, Voges-Proskauer and Citrate utilization

IREC - Institutional Research Ethics Committee

KDB - Kenya Dairy Board

KEMRI - Kenya Medical Research Institute

MHA - Muller Hinton agar

NACOSTI - National Commission for Science, Technology, and Innovation

PCR - Polymerase Chain Reaction

TBC - Total Bacteria Count (TCC)

TCC - Total Coliform Count (TCC)

UK - United Kingdom

UON - University of Nairobi

UPGMA - unweighted pair group arithmetic mean

WHO - World Health Organization

Declarations

Ethical approval

This study's ethical approval was obtained from the International Livestock Research Institute Institutional Research Ethics Committee (project reference: ILRI-IREC2014–04/1). ILRI IREC is accredited by the National Commission for Science, Technology and Innovation (NACOSTI) in Kenya. Ethical approval was also obtained from the Royal Veterinary College ethics committee (project reference: URN 2013 0084H).

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Consent for publication

All authors give permission to proceed with publication of this research

Availability of data and materials

Datasets generated and analysed during the current study are available in the data repository of the University of Liverpool available at (88).

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Authors' contributions

Stella Kiambi and Eric Fevre designed the study and data collection tools. Stella Kiambi collected data; Stella Kiambi, Joshua Onono, John Maina and Eric Fevre drafted the manuscript. Stella Kiambi, Gabriel Aboge, Nduhiu Gitahi developed the culture and isolation standard procedures, facilitated culture and isolation and interpreted results. John Maina, Gabriel Aboge, Beatrice Muchira, and Stella Kiambi conducted molecular analysis, interpreted results. All authors read, commented on, and approved the final manuscript for publication.

Conflict of interest

None

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Figures

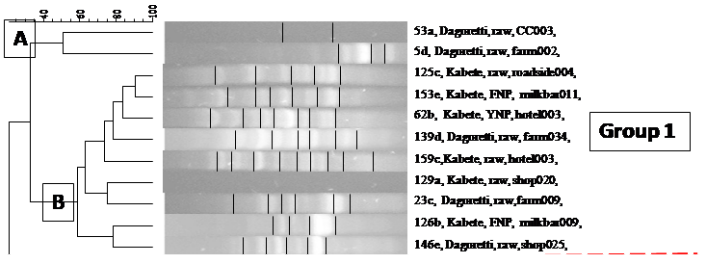

Figure 1

The dendrogram presents a set of 46 diverse *Escherichia coli* isolates from milk samples collected in Nairobi (Kasarani, Dagoretti and Kabete) in Kenya. Samples of fermented non-packed (FNP), fermented packed (FP), pasteurized and raw milk sold at the milk vending machine (ATM), roadside, hotel and shops were collected and analyzed to assess diversity of *Escherichia coli* contaminants. FNP represents home-made fermented milk and FP is processed fermented milk. The first column on the metadata section of the dendrogram represents unique identification code of the isolates.

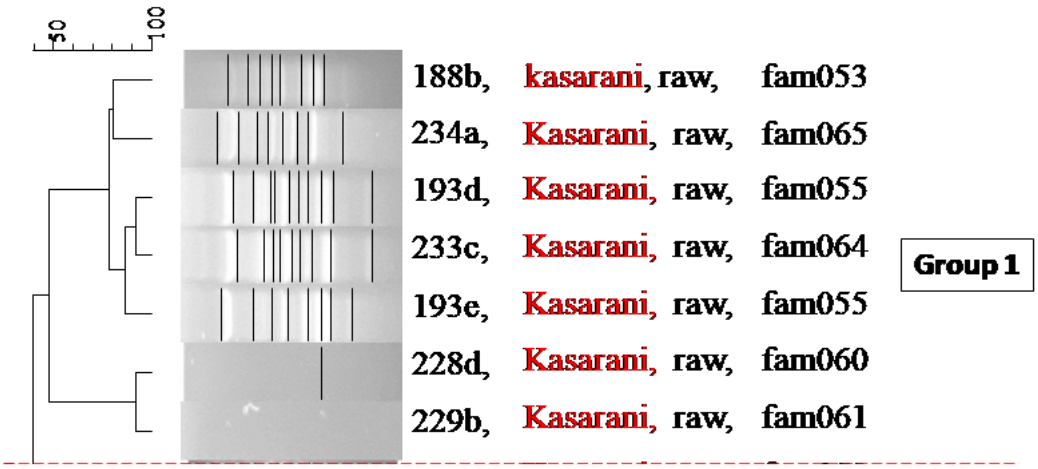

Figure 2

The dendrogram was generated from selected *Escherichia coli* isolates from raw milk samples collected in various farms in Kasarani area. Isolate from the same farm were given the same identification number then letter a-e added for the different isolates. Fingerprint analysis of the 13 *E. coli* isolates from milk samples collected in 9 farms in Kasarani, Nairobi showed two major groups of clusters.

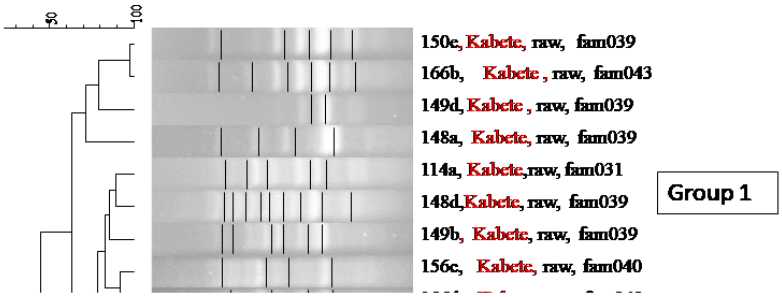

Figure 3

Raw milk samples from 11 farms in Kabete area, Kenya were collected and analysed to establish the genetic diversity of *Escherichia coli*isolates**.** A dendrogram based on banding patterns of 29 *Escherichia coli* showed four major groups of isolates clustering. The dendrogram showed no major indication of clustering of the isolates from same farm in Kabete.

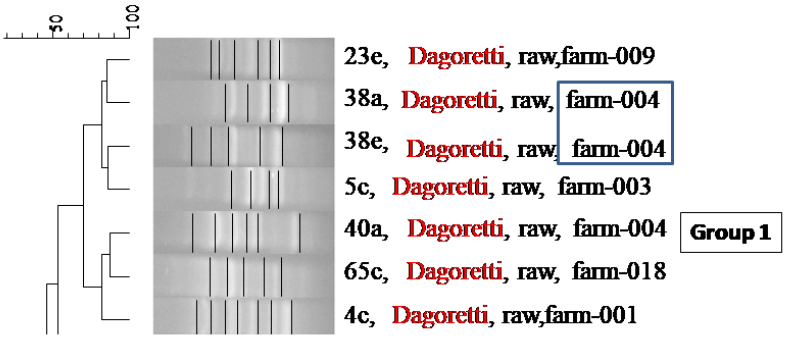

Figure 4

Escherichia coli isolates from milk samples collected in seven farms in Dagoretti, Nairobi were subjected to fingerprint analysis to establish diversity and possible clonal expansion. The 22 *Escherichia coli* analyzed clustered in three major clusters (depicted as group 1, 2, 3) on the dendrogram.