Abstract

Calf diarrhea remains the major health challenges in cattle herds and is the most common cause of morbidity and mortality in neonatal calves. A cross sectional study was conducted from November 2018 to October 2019 to determine the prevalence of E. coli infection in diarrheic calves, investigate potential risk factors of the infection and to determine the antimicrobial susceptibility pattern of E. coli isolates in dairy farms of Holeta and its surrounding area, central Ethiopia. A total of 278 diarrheic calves less than three months of age were purposively selected for this study. Fecal samples collected directly from the rectum of calves were subjected to bacteriological culture and biochemical tests to isolate and identify E. coli. The overall prevalence of E. coli infection in diarrheic calves was found to be 83.5% (232/278×100). The Odds of isolate rates of E. coli for age group 5–8 week was 5.67 times higher as compared to 9–12 week of age (95% CI of OR: 1.18–27.31, P< 0.031). The odds of E. coli isolate rates from diarrheic calves was lower in calves with mucoid diarrhea as compared to those with bloody diarrhea (OR=0.06; 95% CI, 0.01–0.54; P=0.012). The isolate rates of E. coli was 14.5 times higher in calves that received cesaltrum within 6–12 hour than calves that received at their early life in less than 6 hrs (95% CI: 3.53–60.17, P=0.000). Similarly, the isolate rates of E. coli was 29.5 times higher in calves that received cesaltrum after 12 hours of life than calves that received cesaltrum within the first 6 hrs of life (95% CI: 6.77–128.9, P=0.000). The likelihood of isolate rates of E. coli infection is highly prevalent in diarrheic calves < 3 months of age, the majority of which were highly susceptible to Gentamicin (81.82%), sulphamethoxazole (78.79%) and Ciprofloxacin (75.76%), and showed resistance against Streptomycin (81.82%), Tetracycline (60.61%), Ceftriaxone (60.60%) and Cefoxitin (54.55%). It was concluded that E. coli infection is highly prevalent in diarrheic calves < 3 months of age, the majority of which were highly susceptible to Gentamicin, Sulphamethoxazole and Ciprofloxacin antibiotics. Moreover, dairy farms should implement good calf management and hygienic practices including feeding of sufficient quantity of colostrum within the first 6 hrs of birth to prevent E. coli infection.

INTRODUCTION

Newly born calves represent an important source of animal production for either meat or breeding worldwide. Diarrhea is one of the very common disease syndromes in neonatal calves in different countries, causing huge economic and productivity losses to bovine industry worldwide [1]. Calf diarrhea results from multifactorial including Neonatal Calf Diarrhea [NCD] as well as non-infectious factors related to the animals management flaws; inadequate nutrition, exposure to severe environment, insufficient attention to new born calves, or a combination of these [2]. Calves are at greatest risk of developing diarrhea within the first 24 month of life and the incidence of diarrhea decreases with age [3].

Several enteropathogens are implicated in neonatal calf diarrhea, the relative prevalence of which varies geographically; the most prevalent infections causing calf diarrhea include viruses (coronavirus and rotavirus), protozoa (Cryptosporidium spp) and bacteria (Escherichia coli, Clostridium perfringens, Salmonella) [2,4]. In most cases the causes of neonatal calf diarrhea outbreaks are multifactorial and associated with more than one of these agents [5,1]. Among the bacteria, Escherichia coli is the most common primary agent causing calf diarrhea and results in huge economic loss with high morbidity (75%) and mortality (60%) in the cattle industry worldwide [4–6,8]. Escherichia coli are motile Gram-negative bacilli that falls within the family Enterobacteriaceae and usually found as normal inhabitant in the gastrointestinal tract of animals and human. It colonizes the infant gastrointestinal tract and can cause severe gastrointestinal illness [9,10]. Many strains of the bacterium are harmless to the calf, but certain strains that acquire virulence genes can cause moderate to severe scours and even death [11].

The strains inducing gastrointestinal disease are known as Diarrheagenic E.coli (DEC). DEC are subdivided seven pathotypes based on their virulence properties: Enteropathogenic E.coli (EPEC), Enterotoxigenic E.coli (ETEC), Enterohemorrhagic (EHEC), Enteroinvasive E.coli (EIEC), Enteraggregative E.coli (EAggEC), Enterogrouping E.coli (EAggEC) and Enteroadherent E.coli (EAdEC) [5,9].
Central E. coli

d = desired absolute precision.

Required sample size

Sample Size determination

The sample size for this study was estimated using the formula described by [22] with a desired absolute precision of 5%, confidence level of 95% and expected prevalence of 89.9% [20].

$$n = \frac{1.96^2 P_{\text{exp}} (1 - P_{\text{exp}})}{d^2},$$

where:

- $n$ = Required sample size
- $P_{\text{exp}}$ = Expected prevalence
- $d$ = Desired absolute precision.
Accordingly, the calculated sample size for estimating prevalence in purposive sampling was 140. However, in order to make a prevalence estimate more precise, the sample size was inflated two times and was set to 278. Hence, a total of 278 calves were sampled for the study.

**Sample collection and Laboratory processing**

**Sample collection:** Fecal samples were collected aseptically from 278 calves from eighteen farms and kept separately in peptone water. The samples were collected directly from rectum by manual stimulation using disposable latex glove and samples were cooled on ice packs and transported to Holeta Agricultural research center, Department of animal health and National animal health Diagnostic and investigation center (NAHDIC) laboratory for isolation of *E. coli.* The samples were processed as soon as possible after collection/if not keeping in refrigeration at +4°C.

**Bacterial Culture and isolation:** All medium used were prepared according to the manufacturer instruction. Isolation of *E. coli* was conducted following standard procedures described in Quinn et al. [32]. Fecal samples were cultured on MacConkey agar which selectively grows the members of Enterobacteriaceae and incubated at 37 °C for 24-48hrs. Colonies showing characteristic lactose fermenting and having pink color colonies grouped under the species *E. coli.* The presence of growth on MacConkey agar was used as primary criteria to proceed for isolation and identification of *E. coli.*

Furthermore, the colony characteristics observed on MacConkey agar was used to classify suspected bacteria isolated into two groups: lactose fermenters and non-lactose fermenters. Suspected *E. coli* colonies were presumptively identified by their lactose fermenting character (pink colonies) were further sub-cultured on Eosin methyl blue (EMB) agar medium to selectively identify *E. coli.* The characteristic colonies of *E. coli* were identified on EMB based on their green metallic sheen or blue-black to brown color. All isolated colonies were preserved on nutrient agar for further biochemical tests. All isolates were stained by Gram stain to determine the cell morphology, gram reaction and purity of the isolates under the oil immersion objective (X100 magnification).

**Biochemical tests**

Biochemical tests used for differentiation of Enterobacteriaceae use different enzymes. The isolated bacterial pathogens with specific cultural characteristics of *E. coli* maintained on nutrient agar used for further biochemical tests such as Methyl red (MR), Vogues Proskauer (VP) test, Indole test, citrate utilization test, catalase test and Triple Sugar Iron (TSI) test.

**Anti-microbial susceptibility test of *Escherichia coli***

Antimicrobial susceptibility was done by using Kirby Bauer’s disc diffusion method according to performance standards of CLISI clinical and laboratory standards institute.

Briefly, after pre-inoculation of pure bacterial colony in Tryptone soy broth/or saline water and adjustment of turbidity to a 0.5 McFarland turbidity standard, bacterial suspensions are plated as a full lawn onto freshly prepared Mueller-Hinton agar plates using sterile swabs. The antimicrobial discs of streptomycin (10μg), Cefoxitin (30μg), Ceftriaxone (5μg), Nitrofurantoin(30μg), Ciprofloxacin (5μg), Gentamicin (10μg), sulphamethoxazole (100μg) and tetracycline (30μg), were selected to test susceptibility pattern of *Escherichia coli.* The disks

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**Figure 1 Map of study area.**

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were placed on the surface of the inoculated agar plates at about 2cm apart and the plates were incubated at 37 °C for 18 to 24 h. After incubation, the antibiotic Inhibition Zone Diameters (IZD) were measured in millimeter using a caliper and interpreted as susceptible, intermediate and resistant as described by the CLSI [23].

**Questionnaire survey**

A pretested questionnaire was administered to dairy farm owners to assess the general calf husbandry practices at their respective farms. The questionnaire includes all practices in dairy farms including calf health care, hygiene, health problems, colostrum feeding time and method as well as types of diarrhea that affect the growth of calves.

**Data analysis**

All collected data were coded and entered in to the MS-Excel spread sheet and then transferred to the STATA software (STATA version 14) for analysis. Descriptive statistics was used to summarize the generated data. Prevalence of *E. coli* infection was calculated as the number of positive samples divided by the total number of samples examined and multiplied by 100. The univariate and multivariate logistic regression tests were used to calculate the odds ratio (or) of different risk factors. A p-value <0.05 was considered to be indicative of a statistical significant difference.

**RESULTS**

**Prevalence of Escherichia coli infection**

The overall prevalence of *Escherichia coli* infection in diarrheic calves less than 3 months of age in the study area was found to be 83.5% (232). A statistically significant difference (P=0.000) was observed among the three age groups being young calves less than 4weeks showed higher odds of infection (OR=8.35; 95% CI, 3.18–21.86; P=0.000) as compared to other age groups. The prevalence of *Escherichia coli* infection was higher (85.26%) in the Exotic breed calves as compared to in the local breed (57.69%) and cross breed (78.57%). However no statistically significant variation (P>0.05) was observed in prevalence of the infection among the three breeds group (Table 1).

Out of the total calves raised in poor, moderate and good hygienic barns, 90.43%,82.61% and 73.24%, were positive for *E. coli* infection, respectively. The difference in the prevalence of *E. coli* among the three hygienic barn categories was also statistically significant ($\chi^2=9.47, P=0.009$).

**Risk factors**

The univariable logistic regression analysis showed significant association between infection rates of *E. coli* and age (P= 0.000), size of farm (P= 0.001), type of diarrhea (P=0.000), time of colostrum feeding (P= 0.000), method of colostrum feeding (P=0.000) and house hygiene and sanitation (P= 0.003), whereas, sex, breed and BCS have not shown significant association (Table 1).

Multivariate logistic regression analysis revealed that age, type of diarrhea, time of colostrum feeding and methods of colostrum feeding are independent predictors of fecal *E. coli* isolate rates from diarrheic calves (P≤0.05)(Table 1).

**Antimicrobial susceptibility profile of *E. coli* isolates**

The susceptibility of *E. coli* isolates (N=33) was tested to 8 commonly used antimicrobials. The results revealed that *E. coli* isolates were highly susceptible to Gentamicin (81.82%), sulfamethoxazole (78.79%) and Ciprofloxacin (75.76%), and were resistant against streptomycin (91.82%), tetracycline (60.61%), Ceftiraxone (60.60%) and Cefoxitin (54.55%) (Table 2).

**DISCUSSION**

Results of the present study demonstrated the presence of *E. coli* in high proportion of the diarrheic calves. The prevalence of *E. coli* obtained in this study, 83.5%, is in agreement with previous findings of 89.9% prevalence in Ethiopia reported by Zelalem et al. [20], 86.7% in Iran by Pourtaghi et al. [24] and 84.3% in central highlands of Ethiopia by Lema et al. [25]. However, the current result is higher than the 76% prevalence report in Bangladesh by Paul et al. [26], 73.12% prevalence in USA by Foster and Smith [10], 70.7% in Ethiopia by Yeshiwas and Fentahun [27], 44% in Bangladesh by Masud et al. [28] and the 43.1% in Addis Ababa, Ethiopia by Dereje [29]. Such variation may be attributed to difference in climatic conditions, sample size, sampling strategies, feeding managements, hygienic measures, age of the sampled animals as well as farm size [1].

A significant association was observed between age of calves and *E. coli* infection in this study which is in harmony with findings of Yeshiwas and Fentahun [27], Aggernesh [30], Temesgen [31] and Dereje [29] who stated that calves aged between 1-30days, particularly during the first week of life were at greater risk of diarrhea and that risk decreases with age. The reason for this association is related to the ability *E. coli* bacteria to colonize the sterile intestine at early stage of neonate and higher susceptibility of newborn calves than adults, especially if they received low amount of colostrum [32]. Moreover, Godden [33], Mellor and Stafford [34] stated that the structure of the bovine placenta can impede easy acquisition of immune globulins to the unborn calves during pregnancy and consequently, calves may be borne without circulating protective antibodies rendering them more susceptible to different pathogens during their early life.

The current study demonstrated that calf diarrhea was higher in medium and large sized dairy farms as compared to small sized dairy farms. This finding is in consistent with a study report by Yeshiwas and Fentahun [27]. A marked increase in population density commonly results in an increase in the incidence of infectious diseases and mortality [35,36]. Herd size by itself has not a biological effect on the calf health; rather, it may be a measurement of other factors like management and care for calves. The other possible reason for the association of herd size and calf morbidity could be that adequate time may elapse between successive births in small herd size farms, which will reduce the concentration of infectious agents in the calf-rearing environment [27,35]. The present study showed that *E. coli* isolate rate was significantly associated with type of diarrhea, higher proportion of the calves showed watery diarrhea as compared to mucoid, bloody or mixed types of diarrhea, which is in agreement with previous findings of Aggernesh [30], Dereje [29] and Yeshiwas and Fentahun [27].
Table 1: Logistic regression analysis of potential risk factors.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Category</th>
<th>Univariable OR (95% CI)</th>
<th>P-value</th>
<th>Multivariable OR (95% CI)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age</td>
<td>&lt;4 week</td>
<td>8.35 (3.18, 21.86)</td>
<td>0.000*</td>
<td>1.34 (0.28, 6.32)</td>
<td>0.715</td>
</tr>
<tr>
<td></td>
<td>5-8 week</td>
<td>2.3 (0.92, 5.73)</td>
<td>0.074</td>
<td>5.67 (1.18, 27.31)</td>
<td>0.031*</td>
</tr>
<tr>
<td></td>
<td>9-12 week</td>
<td>Ref</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Breed</td>
<td>Local</td>
<td>Ref</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Cross</td>
<td>1.58 (0.68, 3.65)</td>
<td>0.287</td>
<td>0.46 (0.08, 2.71)</td>
<td>0.396</td>
</tr>
<tr>
<td></td>
<td>Exotic</td>
<td>1.12 (0.39, 3.16)</td>
<td>0.829</td>
<td>3.39 (0.28, 40.15)</td>
<td>0.332</td>
</tr>
<tr>
<td>Sex</td>
<td>Female</td>
<td>0.60 (0.31, 1.14)</td>
<td>0.121</td>
<td>1.06 (0.37, 3.02)</td>
<td>0.905</td>
</tr>
<tr>
<td></td>
<td>Male</td>
<td>Ref</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BCS</td>
<td>Good</td>
<td>Ref</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Medium</td>
<td>0.83 (0.39, 1.75)</td>
<td>0.628</td>
<td>0.46 (0.15, 1.44)</td>
<td>0.186</td>
</tr>
<tr>
<td></td>
<td>Poor</td>
<td>2.00 (0.84, 4.82)</td>
<td>0.117</td>
<td>1.64 (0.44, 6.12)</td>
<td>0.465</td>
</tr>
<tr>
<td>Farm size</td>
<td>Small</td>
<td>Ref</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Medium</td>
<td>1.97 (0.77, 5.05)</td>
<td>0.158</td>
<td>0.26 (0.04, 1.69)</td>
<td>0.159</td>
</tr>
<tr>
<td></td>
<td>Large</td>
<td>3.25 (1.61, 6.61)</td>
<td>0.001*</td>
<td>0.36 (0.07, 1.94)</td>
<td>0.239</td>
</tr>
<tr>
<td>Type of diarrhea</td>
<td>Bloody</td>
<td>Ref</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Watery</td>
<td>1.23 (0.52, 2.92)</td>
<td>0.637</td>
<td>1.69 (0.46, 6.17)</td>
<td>0.424</td>
</tr>
<tr>
<td></td>
<td>Mucoïd</td>
<td>0.05 (0.10, 0.22)</td>
<td>0.000*</td>
<td>0.06 (0.01, 0.54)</td>
<td>0.012*</td>
</tr>
<tr>
<td></td>
<td>Mixed</td>
<td>0.36 (0.10, 1.34)</td>
<td>0.129</td>
<td>0.14 (0.01, 1.56)</td>
<td>0.110</td>
</tr>
<tr>
<td>Time of</td>
<td>&lt; 6 hour</td>
<td>Ref</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Colostrum</td>
<td>6-12 hour</td>
<td>7.28 (3.04, 17.43)</td>
<td>0.000*</td>
<td>14.5 (3.5, 60.17)</td>
<td>0.000*</td>
</tr>
<tr>
<td>feeding</td>
<td>&gt;12 hour</td>
<td>14.03 (6.08, 32.3)</td>
<td>0.000*</td>
<td>29.5 (6.77, 128.9)</td>
<td>0.000*</td>
</tr>
<tr>
<td>Method of</td>
<td>Suckling</td>
<td>Ref</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Colostrum</td>
<td>Hand feeding</td>
<td>9.92 (4.90, 20.06)</td>
<td>0.000*</td>
<td>40.4 (7.78, 210)</td>
<td>0.000*</td>
</tr>
<tr>
<td>House hygiene</td>
<td>Good</td>
<td>Ref</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>and Sanitation</td>
<td>Moderate</td>
<td>1.74 (0.82, 3.68)</td>
<td>0.151</td>
<td>1.28 (0.40, 4.15)</td>
<td>0.671</td>
</tr>
<tr>
<td></td>
<td>Poor</td>
<td>3.45 (1.53, 7.79)</td>
<td>0.003*</td>
<td>3.24 (0.91, 11.60)</td>
<td>0.070</td>
</tr>
</tbody>
</table>

Table 2: Antimicrobial susceptibility test of *E. coli* isolates (N=33).

<table>
<thead>
<tr>
<th>Antimicrobials</th>
<th>Susceptible N (%)</th>
<th>Intermediate N (%)</th>
<th>Resistant N (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cefoxitin</td>
<td>6(18.18)</td>
<td>9(27.27)</td>
<td>18(54.55)</td>
</tr>
<tr>
<td>Ceftriaxone</td>
<td>4(12.12)</td>
<td>9(27.27)</td>
<td>20(60.60)</td>
</tr>
<tr>
<td>Ciprofloxacin</td>
<td>25(75.76)</td>
<td>6(18.18)</td>
<td>2(6.06)</td>
</tr>
<tr>
<td>Gentamicin</td>
<td>27(81.82)</td>
<td>3(9.09)</td>
<td>3(9.09)</td>
</tr>
<tr>
<td>Nitrofurantoin</td>
<td>12(36.36)</td>
<td>16(48.49)</td>
<td>5(15.15)</td>
</tr>
<tr>
<td>Streptomycin</td>
<td>2(6.06)</td>
<td>4(12.12)</td>
<td>27(81.82)</td>
</tr>
<tr>
<td>Sulphamethoxazole</td>
<td>26(78.79)</td>
<td>3(9.09)</td>
<td>4(12.12)</td>
</tr>
<tr>
<td>Tetracycline</td>
<td>5(15.15)</td>
<td>8(24.24)</td>
<td>20(60.61)</td>
</tr>
</tbody>
</table>

The occurrence of *E. coli* was significantly associated with colostrum feeding time of the calves; the highest prevalence of *E. coli* was in calves that were fed colostrum after 12 hours of age due to low level of immunoglobulin. Calves fed with colostrum at less than 6 hour of age had lower odds of *E. coli* isolates than those fed colostrum after 12 hours of age. This results in agreement with reports of Razzaque et al. [37] and Yeshiwas and Fentahun [27]. Each hour of delay in colostrum ingestion in the first 12 hours of age increases the chance of calves becoming ill by 10%. Delaying colostrum intake is known to decrease intestinal immunoglobulin and fat-soluble vitamins absorption and there is no immunoglobulin absorption into the circulation after 24 hours due to closure of calf’s gut [38,39]. The time between birth and the first feeding is the prime factor for the failure of passive transfer of colostral immunity. Several studies had reported that calf mortality is significantly higher in those that got colostrum late after birth than those that got colostrum soon after birth [27]. This indicates that administering the first colostrum with proven quality and sufficient amount of immunoglobulins as soon as birth is critical to ensure successful passive transfer and health of neonatal calves.

In the current study, the occurrence of *E. coli* infection was higher in calves that fed colostrum by hand feeding method as compared to those fed by suckling method. Similarly, Yeshiwas and Fentahun [27] reported that failure of passive immunity transfer in bottle feeding is higher than in naturally suckled calves. This might be attributed to refusing and inability of calves
to ingest enough quantity of colostrum during hand feeding. Furthermore, risk of contamination of the colostrum with environmental pathogens is very high. Contaminated colostrum is one of the earliest potential exposures to infectious agents that cause diarrhea and septicemia in newborn calves [39].

Results of this study revealed a significant statistically association between E. coli isolate rates and calf house hygiene and sanitation. Calves with poor hygiene and sanitation were more likely to acquire E. coli infection than calves in good house hygiene practices. This finding is comparable with the reports of Bazeley [38] that reported housing types and poor hygiene as risk factors for calf diarrhea. Similarly Yeshiwas and Fentahun [27] indicated that the occurrence of E. coli was high in muddy or wet livestock floor. Fecal contamination is more likely to occur during feeding, watering and handling from poor hygienic and unsanitary house and also increase risk of dissemination of Escherichia coli to newborn calves. Dairy cattle housed in barns with flush-type manure removal systems were more likely to have E. coli than animals housed in barns where manure was removed by scraping [40].

The present study showed that 81.82% of the E. coli isolates were susceptible to Gentamicin followed by 78.79% susceptibility to sulphonamide and 75.76% susceptibility to Ciprofloxacin. This high susceptibility might be due to uncommonly usage/ unavailability of these drugs for animal disease treatment in Ethiopia. On the other hand, Streptomycin, Tetracycline and Ceftriaxone showed the highest rates of resistance with 81.82%, 60.61% and 60.60%, respectively. Comparable findings have been reported by Bagre et al. [41] from Burkina Faso, in which all E. coli isolates were 100% susceptible to gentamicin and 15% resistant to sulphonamide. The antibiogram study done by Yeshiwas and Fentahun [27] revealed that the E. coli isolates were highly sensitive to tetracycline, sulphonamide, chloramphenicol, streptomycin, oxacillin; less sensitive to amoxicillin, cefazidime, nitrofurantoin, kanamycin and resistance to cepotaxime, vancomycin. The recorded antimicrobial resistance of E. coli to streptomycin, tetracycline and cefotaxime in the current study might be as a result of suboptimal, prolonged and misuse use of antimicrobials for prophylaxis and treatment of infection. Antimicrobial resistance emerges from the use of antimicrobials in animals and human, and the subsequent transfer of resistance genes and bacteria among animals, humans, animal products and the environment [42].

CONCLUSION AND RECOMMENDATIONS

The current study revealed somewhat high prevalence of E. coli infection in diarrheic calves in dairy farms of Holota and its surrounding areas, Central highlands of Ethiopia. Age of calves, farm size, time and method of colostrum feeding, as well as house hygiene and sanitation were the most determinant factors that predispose calves to E.coli infection. Isolates of E.coli in the study area were highly susceptible to gentamicin, sulphonamide and Ciprofloxacin and resistant to streptomycin, tetracycline and ceftriaxone.

- Based on above findings, the following recommendations are suggested: Implementation of good calf management practice is highly advised particularly feeding of sufficient amount of colostrum shortly after birth before the gut closure is completed.
- Preventive approaches against E. coli infection directed towards management risk factors should best strengthened to minimize morbidity and mortality associated losses.
- Veterinarians should pay attention to E. coli resistant antibiotics while prescribing remedies for the treatment of colibacillosis in calves
- Further investigation on the prevalence of E. coli should be under taken in order to design cost effective control measures.

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