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Comparative Study on the Prevalence of *Escherichia coli* and it's Antibiogram in *Saum* from Three Different Storage Places in Household at Mizoram

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ABSTRACT

Background: *Saum* is a very important traditional food item of the Mizo society in Mizoram, North East India; but there is scanty scientific study and reports on *Saum*. The present study has been conducted to study and compare the prevalence of *E. coli* in *Saum* from three different storage places such as storage/keeping *Saum* under the sun (24-38°C) during hot part of the day, inside the refrigerator (3-4°C) and near the cooking fire (30-50°C) at home.

Methods: The study was conducted from May, 2022 to November, 2022. A total of 105 *Saum* samples were collected from three different storage places comprising of 35 samples from each storage places and then analysed for the presence of foodborne pathogen *E. coli* and it's virulence genes (*stx*$_1$, *stx*$_2$, *elt, est* genes) by conventional method and PCR. All the *E. coli* isolates were subjected to 12 different antibiotics for antimicrobial resistance pattern by disc diffusion method.

Result: The overall prevalence of *E. coli* from 105 samples was 7.62% (8/105) comprising of 5.71% (2/35) from the storage place under the sun, 2.86% (1/35) from the refrigerator and 14.29% (5/35) near the cooking fire and no presence of virulence genes of *E. coli* was detected in the present study. The antimicrobial resistance pattern of all the 8 *E. coli* isolates showed the highest resistance to Cefazolin (100%) followed by Imipenem (37.5%). The present work will be a complementary contribution among Mizo society to know the best and safest storage places of *Saum* in regards to public health point of view.

Key words: *Escherichia coli,* PCR, Prevalence, *Saum,* Virulence genes.

INTRODUCTION

Traditional foods are a significant part of the culture, legacy and identity of people from different parts of the world (Halagarda and Wójciak, 2022). The ethnic people of North-East India are one of the groups with a long history having the habit of transferring different ancient traditional fermented food from generation to generation for thousands of years ago (Deka *et al*., 2021). Among the North-East people of India, the "Mizo" tribe comprising of the majority of the local population of Mizoram had a distinctive customs and ethnicity and they have adapted many traditional food preparation processes from their ancestors. One of Mizo cuisine's most distinctive traditional foods is *Saum*, a fermented pork fat usually cooked with the vegetable *"Bai"* and occasionally eaten as a pickle. This *Saum* is made from caul adipose tissue and it is a semi-dry, sticky and "ripened" fat (De Mandal *et al*., 2018). Traditional meat products are often recognised as safe and nutritious. On the contrary, there are hazards of a microbiological nature linked with some traditional meat products, particularly the fermented ones (Holck *et al*., 2017). As a result, it is critical to confirm the true safety risks and nutritional qualities of traditional meat products. The food-borne pathogen *E. coli* is the most prevalent and is the most easiest to contaminate which is commonly found in warm-blooded animals' lower intestines. Though the majority of *E. coli* strains are often harmless but some of them can result in life-threatening foodborne illnesses. Shiga toxin-producing (STEC) serotype *E. coli* O157:H7, has been identified from a range of foods and causes moderate to severe diarrhoea as well as hemolytic

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uremic syndrome (HUS), which is one of the most prevalent food-borne pathogen (World Health Organization, 2018; Bintsis, 2017). All the Mizo indigenous food products and their processing processes are not well documented so far (Lalthanpuii *et al*., 2015) and among them the most famous traditional food item "*Saum*" of the Mizos in Mizoram, India has scanty scientific studies and reports though it has been used from forebears traditionally and extensively. According to the research conducted by Ralte (2020), the Mizos has the habit of storing their *Saum* in the container in three different storage places such as storing *Saum* under the sun (24-38°C) during the hot part of the day, inside the refrigerator (3-4°C) and near the cooking fire (30-50°C) in the kitchen. There were no reported studies about the comparison for the prevalence of *E. coli* from these three different storage places of *Saum* as *E. coli* is one of the most important indicator organisms of sanitation and its presence in the food items indicates poor sanitary practices (Bachhil *et al.,* 2016). So, the present study was confined to detect and compare for the prevalence of *E. coli* to trace out the best storage places and it's antibiogram from three different storage places of *Saum*.

MATERIALS AND METHODS

The experiment was conducted at the laboratory of Department of Veterinary Public Health and Epidemiology, College of Veterinary Sciences and Animal Husbandary, Central Agricultural University, Selesih, Aizawl, Mizoram, North East India from the month of May, 2022 to November, 2022.

Isolation and identification of *E. coli*

A total of 105 samples were collected from three different storage places namely under the sun, inside the refrigerator and near the cooking fire comprising of 35 no. of samples from each storage places. All the 105 *Saum* samples were processed for the detection of the presence of *E. coli.* Firstly, the isolation and identification of *E. coli* was done as per the standard bacteriological method described by Cowan and Steel (1993). One gram of each sample was mixed with 9 mL of sterile Lactose broth (HiMedia™) in a sterile test tube and then incubated at 37°C for 24 hours at BOD incubator (NSW-India™). The suspected positive tubes containing *E. coli* were selected and a loop-full of enriched culture from each of the positive broths was streaked onto Eosin-methylene blue (EMB) agar (HiMedia™) for confirmation of *E. coli* and were incubated again for 24 hours at 37°C in an incubator. The morphological characteristics of the isolated *E. coli* were studied after staining the fresh culture smears by Gram's staining method and observations were recorded for the identification of each isolates (Quinn *et al*., 1994). The methodology recommended by Quinn *et al*. (2004) was followed for biochemical confirmation. One or two typical suspected colonies was transferred from each EMB plate into test tubes containing nutrient broth (HiMedia™) and incubated at 37°C for 24-48 hours. Following incubation period, a loop full of nutrient broth were streaked on the nutrient agar (HiMedia™) plates and incubated at 37°C for 24-48 hours. The pure isolate colonies were subjected for biochemical confirmation using standard biochemical tests, *viz*., Indole, Methyl red, Voges-Proskauer and Citrate utilization. Further, all the 8 isolates of *E. coli* detected by conventional method was confirmed by PCR.

Molecular detection of virulence genes of *E. coli*

All the *E. coli* isolates positive for *16S-rRNA* species specific gene were screened for the presence or absence of virulence associated enterotoxin genes namely stx_1 and stx_2, *est* and *elt* of *E. coli* using published primer (Imperial Life Sciences India Pvt. Ltd., Gurgaon, Haryana, India) and the PCR protocol for detection of different genes was conducted as per the methodology described by Paton and Paton (1998), Dadie *et al*. (2014) and Moyo *et al*. (2007) respectively as shown in Table 1. Amplification of DNA was performed in a Thermal cycler machine with a pre-heated lid. The details of the various cycling conditions for the species specific gene and different virulent genes were given in Table 2. After completion of PCR reaction, the PCR products were stored at 4°C for further analysis by agar gel electrophoresis. All the amplified PCR products were subjected for agarose gel electrophoresis. 1.5% agarose gel was prepared by boiling 0.9 g agarose in 60 mL of 1× TAE buffer in a conical flask for about 2 minutes in a microwave and after it cooled down 0.2 µl Ethidium bromide was added. Then the molten agarose gel was poured into a casting tray fitted with acrylic comb until the gel was solidified in undisturbed manner. After 20 minutes, the comb was removed and casting tray gel was placed in a submarine gel electrophoresis (Bio Products; UK). The unit of gel electrophoresis was filled with 1× TAE buffer up to the level of 1 mm above the surface gel. About 5 µl PCR product was loaded into each well of the gel in an electrophoresis apparatus for 45 minutes at 70 V/100 mA till the dye reached the last third of the gel and the gel was visualized under UV transilluminator documented by gel documentation system (Alpha imager; Proteinsimple; California, USA). 100 bp DNA ladder have been used as reference to compare the size of amplified products. The presence of different virulence genes *E. coli* was checked by visualizing the specific band size (bp) on Agarose gel and interpreted with the help of gel documentation system.

Detection of antibiotic sensitivity and resistance pattern of *E. coli* isolates

All the *E. coli* positive strains were analysed to antibiotic sensitivity by disc diffusion method (Bauer *et al*., 1966) against a panel of 12 antibiotics namely Ampicillin, Amoxyclav, Cefotaxime, Ceftriaxone, Cefazolin, Ciprofloxacin, Co-Trimoxazole, Gentamicin, Imipenem, Norfloxacin, Ofloxacin and Tetracycline as per the Clinical and Laboratory Standard Institute guidelines (CLSI, 2020). Positive isolates were inoculated into 5 mL of sterile Luria Bertani (LB) broth under constant shaking at 37°C for overnight. The overnight broth cultures were spread uniformly over Mueller Hinton agar

plates (HiMedia™) with the help of sterile spreaders. The plates were allowed to dry for 10-15 minutes to absorb the liquid. Antibiotic discs were placed on inoculated agar surface at about 2 cm apart by using sterile forceps. The plates were incubated at 37°C overnight and diameter of the zones of inhibition was measured (CLSI, 2020).

Statistical analysis

The data obtained were analysed using statistical package SPSS version 27.0.

RESULTS AND DISCUSSION

Out of the total 105 samples collected a total of 8 isolates of *E. coli* were obtained comprising of 2 isolates from the storage place of *Saum* kept under the sun, 1 isolate was obtained from inside the refrigerator and 5 isolates from storage place near the cooking fire. All the 8 isolates exhibited metallic sheen on Eosin Methylene Blue (EMB) agar with gram negative bacteria by gram staining method and the biochemical tests showed positive to Indole production test and Methyl Red (MR) test but negative to Voges-Proskauer (VP) and citrate utilization test. The overall prevalence of *E. coli* from 105 samples was recorded to be 7.62% (8/105) comprising of 5.71% (2/35) from the storage place under the sun, 2.86% (1/35) from refrigerator and 14.29% (5/35) near cooking fire storage place. The details on the prevalence of *E. coli* have been presented in Table 3 .

Statistical analysis revealed that there was significant differences ($p<0.05$) between the prevalence of *E. coli* from the storage places of *Saum* near the fire to that of the storage places under the sun and inside the refrigerator but there was no significant differences ($p>0.05$) between the prevalence of *E. coli* from the storage places of *Saum* under the sun to that of the refrigerator storage place. The present finding was in agreement with the finding of Zhang *et al.* (2015) who reported 6.90**%** (10/145) of *E. coli* from fresh raw pork meat. In contrast to the present study, a higher prevalence rate of *E. coli* 15% (30/200) was reported by Lallawmkimi *et al.* (2021) from 200 samples of smoked pork sold in local markets of Aizawl, Mizoram, India and the same rate of *E. coli* prevalence was reported by Azuamah *et al.* (2018) with 15.60% (93/596) from red meat. A slightly higher prevalence of *E. coli* with 23.33% (28/120) was reported by Ralte (2020) from fermented pork product (*Saum*) procured from different parts of Mizoram, India. This might be due to the presence of high contamination level of raw materials with high initial micriobial load, poor hygiene practices during processing and high temperatures (>15°C) by malfunction in the processing lines. In contrast to the present study, Wei *et al.* (2006) reported a lower prevalence rate of *E. coli* obtained from ready to eat food products stored in the refrigerator and room temperatures showed the prevalence rates of *E. coli* with 3% (1/40) and 2% (1/44), respectively. Gamal *et al.* (2020) also reported a low prevalence rate of *E. coli* from sausage 10.0% (5/50). All these low prevalence rate of *E. coli* reported by these researchers might be due to the proper hygienic status of the butcher, systematic sanitation education systems of employee, proper cleanliness of utensils and equipment and continuous monitoring of the microorganisms.

The entire 8 *E. coli* isolates positive for *16S-rRNA* gene screened for the presence of virulence genes *viz*., stx_1, stx_2, *elt, est* gene by using the PCR showed negative results as shown in Table 4. The present finding was in agreement

Table 1: Oligonucleotide primers to be used for detection of species specific gene and virulence associated genes of *E. coli* by PCR.

Target gene	Primer sequence (5′-3′)	Base pair	Reference
16S-rRNA	F: GAC CTC GGT TTA GTT CAC AGA R: CAC ACG CTG ACG CTG ACCA	585	Candrian *et al.* (1991)
stx1	F: ATAAATCGCCATTCGTTGACTAC R: AGAACGCCCACTGAGATCATC	180	Paton and Paton (1998)
stx2	F: AGAACGCCCACTGAGATCATC R: TCGCCAGTTAATCTGACATTCTG	253	Paton and Paton (1998)
est	F: CCA TCA ACA CAG TAT ATC CGA R: GGT CGC GAG TGA CGG CTT TGT	111	Dadie *et al.* (2014)
elt	F: CTC TAG GTG CAC ACG GAG C R: CCA TAC TGA TTG CCG CAA T	321	Moyo *et al.* (2007)

Table 2: Thermal cycling conditions for detection of species specific gene and various virulence genes of *E. coli* isolates.

Stages	*16S-rRNA*	stx_1	stx_2	*est*	*elt*
Initial denaturation	95°C for 5 min	95°C for 5 min	95°C for 5 min	95°C for 5 min	95°C for 5 min
Denaturation	94°C for 45 sec	94°C for 45 sec	94°C for 45 sec	94°C for 45 sec	94°C for 45 sec
Annealing	59°C for 45 sec	61°C for sec	61°C for 45 sec	56°C for 45 sec	61°C for 45 sec
Elongation	72°C for 45 sec	72°C for 45 sec	72°C for 45 sec	72°C for 45 sec	72°C for 45 sec
Final extension for 1 cycle	72°Cfor 6 min	72°Cfor 6 min	72°Cfor 6 min	72°Cfor 6 min	72°Cfor 6 min
No. of cycles	34	34	34	34	34

with the finding of Ralte (2020) who reported the absence of virulence gene of *E. coli* of stx_1, stx_2, *elt* in *Saum* samples from different part of the Aizawl, Mizoram, India. Another researcher Bardasi *et al.* (2017) reported that the absence of stx_1 and stx_2 genes from ready- to- eat pork (RTE) products in Italy. This might be due to the reasons that *E. coli* might be destroyed by heat treatment in the process of preparation. According to World Health Organisation *E. coli* especially STEC is destroyed by thorough cooking of foods until all parts reach a temperature of 70°C or higher (WHO, 2018). In contrast to the present finding Ralte (2020) reported the presence of virulence gene of *E. coli* of *est* gene with 17.86% (5/28) in *Saum* samples from different part of the Aizawl, Mizoram, India which was absent in the present study. The absence of the virulence genes in the present study might be due to the reason that the *Saum* sample used in the present study was collected from a single source prepared by an expert person with strict hygienic practices but Ralte (2020) collected the *Saum* sample from different places involving 120 sources (household and market sample) which might resulted to higher chances of evidence for the occurrence of the virulence gene of *E. coli* as the *est* gene of *E. coli* itself is a heat stable organism too. Another researcher Lallawmkimi *et al.* (2021) reported a total of 14 virulence genes of *E. coli* detected with different virulence genes STEC, EPEC and EHEC of *E. coli* with 10 (40%), 1 (4%) and 3 (12%) respectively from smoked pork in Aizawl, Mizoram, India. Some of the researchers like Bardasi *et al.* (2015) reported 19% (41/213) positive of *stx* genes in fresh pork sausages and 2.8% (19/675) positive of *stx* genes in fresh pork sausages in Italy was reported by Ercoli *et al.* (2016). These virulence genes of *E. coli* presence might be due to the excessive handling, improper cleaning of instruments and equipment, a lack or insufficiency of good manufacturing practices and HACCP (Hazard Analysis Critical Control Points) system; cross contamination due to improper preparation of the product, improper heat treatment of the product during processing along with post preparation contamination as well as differences between distinct geographical locations, major climate conditions and husbandry methods that might allow the introduction of several virulence genes of *E. coli* in the product (Cavalin *et al.*, 2018; Rajkhowa and Sarma, 2014).

Table 3: Prevalence of *E. coli* isolated from *Saum* sample in three different storage places.

Storage places	No of sample analysed	Prevalence of *E. coli* isolates
Sun	35	2 (5.71)[b]
Refrigerator	35	1 (2.86) [b]
Fire	35	5 (14.29)[a]
Total (N=105)	105	8 (7.62)*

a, b Differ significant difference.

*Denotes significant difference ($p \leq 0.05$).

Overall, the *E. coli* isolates showed highest resistance to Cefazolin (100%) followed by Imipenem (37.5%). The highest sensitive (100%) was observed to 6 antibiotics namely Cefotaxime (100%), Ciprofloxacin (100%), Co-Trimoxazole (100%), Norfloxacin (100%), Ofloxacin (100%), Tetracycline (100%) followed by Ceftriaxone (87.5%), Ampicillin (62.5%), Gentamicin (25%) and Amoxyclav (12.5%). The highest intermediate was showed to Amoxyclav (87.5%) followed by Gentamicin (75%), Imipenem (62.5%), Ampicillin (37.5%) and Ceftriaxone (12.5%) as shown in Table 5. In the present study, Cefazolin (100%) followed by Imipenem (37.5%) has shown resistance against of isolates *E. coli* from the *Saum.* In contrast to the present finding, Hnamte *et al.* (2018) reported that Cefazolin (76.08%) has shown resistance against of *E. coli* from meat based fast foods in Mizoram, India. Another researcher also reported that Imipenem has shown resistance 18.18% from Western zone and 11.11% from Central zone of Mizoram against *E. coli* in *Saum* by Ralte (2020). This might due to over usages of antibiotic or misuses of the different antibiotics for the treatment of many diseases in Mizoram, India. The present study reported 100% sensitive against Cefotaxime (100%), Ciprofloxacin (100%), Co-Trimoxazole (100%), Norfloxacin (100%), Ofloxacin (100%), Tetracycline (100%) followed by Ceftriaxone (87.5%), Ampicillin (62.5%), Gentamicin (25%) and Amoxyclav (12.5%). In contrast to the present finding, Ralte (2020) reported that Ciprofloxacin, Gentamicin, Tetracycline (92.8% each) and Amoxyclav (85.71%), Ceftriazone (21.43%) were sensitive against *E. coli* from *Saum* Mizoram, India. Another researcher Hnamte *et al.* (2018) also reported Norfloxacin (93.47%), Gentamicin (78.26%), Ofloxacin (73.91%), Amoxyclav (69.56%), Co-trimoxazole (67.39%) were sensitive against *E. coli* from meat based fast foods in Mizoram, India. All these might be due to the lower doses of antibiotic drugs, uses of prescribed antibiotics in the treatment of animal and also human study area as well as

Table 4: Prevalence of virulence genes of *E. coli* isolates from *Saum* sample in three different storage places.

Storage places	No of sample analysed	No of *E. coli* isolates	Virulence gene			
			stx_1	stx_2	*elt*	*est*
Sun	35	2	Nil	Nil	Nil	Nil
Refrigerator	35	1	Nil	Nil	Nil	Nil
Fire	35	5	Nil	Nil	Nil	Nil
	Total (N)=105	N=8				

Table 5: Antimicrobial resistance, intermediate and sensitivity of *E. coli* isolates from *Saum* samples in three different storage places.

Antimicrobial agents	Disc content (mcg)	No. of *E. coli* isolates	Reaction according to the zone of inhibition		
			Resistance	Intermediate	Sensitive
Cefotaxime (CTX)	30	8	0 (0.00)	0 (0.00)	8^{a} (100)
Ciprofloxacin(CIP)	30	8	0 (0.00)	0. (0.00)	8^{a} (100)
Co-Trimoxazole (CoT)	25	8	0 (0.00)	0. (0.00)	8^{a} (100)
Norfloxacin (NoX)	10	8	0 (0.00)	0. (0.00)	8^{a} (100)
Ofloxacin (OF)	5	8	0 (0.00)	0 (0.00)	8^{a} (100)
Tetracycline (TE)	30	8	0 (0.00)	0 (0.00)	8^{a} (100)
Ceftriaxone (CTR)	30	8	0 (0.00)	1 (12.5)	7^{ab} (87.5)
Ampicillin (AMP)	10	8	0 (0.00)	3 (37.5)	5^{abc} (62.5)
Gentamicin (GEN)	10	8	0 (0.00)	6 (75)	2^{bc} (25)
Amoxyclav (AMC)	30	8	0 (0.00)	7 (87.5)	1^{c} (12.5)
Imipenem (IMP)	10	8	3 (37.5)	5 (62.5)	0 (0.00)
Cefazolin (CZ)	30	8	8 (100)	0 (0.00)	(0.00)

a, b and c differ significant difference.

adequate awareness regarding misuses of the antibiotics in the population.

CONCLUSION

The best storage place of *Saum* is inside the refrigerator storage place (3-4°C) among the three different storage places of *Saum* (*viz*. sun, refrigerator and near fire) as the lowest prevalence rate of *E. coli* with 2.86% (1/35) was detected in the refrigerator storage place and this shows that the *Saum* sample may be stored in the refrigerator only at post preparation of the product for the improvement of hygienic handling and maintenance of the post preparation of *Saum*. If refrigerator is not available at home in some villages and remote areas, then the *Saum* sample may to be stored/kept under the sun (24-38°C) on every hot part of the day instead of storing the post prepared *Saum* sample near the cooking fire (30-50°C). The present study also concluded that during the process of preparation of *Saum* sample proper heat treatment/cooking of raw pork and good sanitary practices by the producers of *Saum* is very essential which might have lowers the occurrence of *E. coli.* A high antibiotic resistance shown to Cefazolin (100%) by *E. coli* in the present study contributed an importance of judicious use of antibiotics in the treatment of animals and human being in the future.

Conflict of interest

All authors declare that they have no conflict of interest.

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