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## Isolation and identification of gram negative bacteria from street-vended sauce and brand sauce in Dhaka city to evaluate their safety margin

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### Abstract

The aim of this study was to isolate and identify different kinds of gram negative bacteria from street vended sauce and brand sauce in the city of Dhaka to evaluate their safety margin. Nine different sauce samples were collected from different areas of Dhaka city. Four of those samples were brand sauce and five were street sauce. Four types of selective media were used to isolate gram negative bacteria: Mac-Conkey Agar (MAC), Thiosulfate-Citrate-Bile Salts-Sucrose Agar (TCBS agar), Salmonella- Shigella Agar (SS agar) and Eosin-Methylene Blue (EMB). Serial dilution of each sauce sample was performed then samples were cultured on selective media plates by spread plate technique and colony forming units were observed to determine whether their number was below or above the minimum infective dose to evaluate their safety margin. Biochemical tests were performed to identify the isolated colony forming units. Bacteria isolated in different selective media from all the brand sauce samples were far below the minimum infective dose for humans. Bacteria isolated from all Street Sauce samples in the MAC agar and EMB media were found to be more than minimum infective dose. Three of the five Street sauce samples displayed more colony forming units in the SS agar media than the minimum infective dose. In TCBS agar media two of the five Street sauce samples displayed colonies near the minimum infection dose and another one was above. Biochemical tests displayed presence of *Escherichia coli*, *Salmonella typhi*, *Shigella dysenteriae*, *Vibrio cholera* and *Vibrio parahaemolyticus*.

**Keywords:** Gram-negative, spread plate, selective media, biochemical tests

### 1. Introduction

Sauce is liquid, cream or semi-solid food served on or used in preparing other foods. Sauces are not normally consumed by themselves; they add flavor, moisture, and visual appeal to another dish. They are an essential element in cuisines all over the world. Usually sauces are produced either by food manufacturing companies or locally for street-vended food industry [1].

The street-vended food industry provides employment and cheap ready-to-eat meals to a large proportion of the population in developing cities like Dhaka, yet little is known about its role in the transmission of food borne diseases. Because of its wide consumption, street-vended sauces in Dhaka are potential vehicles of food borne diseases [2]. To ensure the safety margin of street vended food products it is necessary to compare them with Brand products [3].

Street vended food contains both gram positive and gram negative bacteria Gram-negative bacteria are more dangerous as disease organisms, because their outer membrane is often hidden by a capsule or slime layer which hides the antigens of the cell and so acts as "camouflage" - the human body recognize a foreign body by its antigens; if they are hidden, it becomes harder for the body to detect the invader. Often the presence of a capsule will increase the virulence of a pathogen. Additionally, Gram-negative bacteria have lipopolysaccharide in their outer membrane [4]. Lipopolysaccharide is an endotoxin which increases the severity of inflammation. This inflammation may be so severe that septic shock may occur. Gram-positive infections are generally less severe because the human body does not contain peptidoglycan, and in fact the human body produces an enzyme called lysozyme which attacks the open peptidoglycan layer of Gram-positive bacteria. Gram-positive bacteria are also much more susceptible to beta-lactam antibiotics, such as penicillin [5].

Common gram negative bacteria in street vended food belong to the genus *Escherichia*, *Vibrio*, *Shigella*, *Salmonella*, *Campylobacter* and *klebsiella* [6].

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The most widely used selective media for isolating gram negative bacteria are MacConkey agar, Thiosulfate-citrate-bile salts-sucrose agar, Salmonella Shigella agar and Eosin methylene blue agar [7].

MacConkey agar is used for the isolation of gram-negative enteric bacteria and the differentiation of lactose fermenting from lactose non-fermenting gram-negative bacteria [8, 9].

Thiosulfate-citrate-bile salts-sucrose agar is a type of selective agar culture plate that is used in microbiology laboratories to isolate *Vibrio* spp. It is highly selective for the isolation of *Vibrio cholerae* and *Vibrio parahaemolyticus* as well as other *Vibrios* [10].

Salmonella Shigella Agar is moderately selective and differential medium for the isolation, cultivation and differentiation of *Salmonella* spp. and some strains of *Shigella* spp. [11].

Eosin Methylene Blue media assists in visual distinction of *Escherichia coli*, other nonpathogenic lactose-fermenting enteric gram-negative rods, and the *Salmonella* and *Shigella* genera [12].

To ensure the safety of a food product it is necessary to maintain the bacterial count under the minimum infective dose. The minimum infective doses of common gram negative bacteria are *Escherichia coli* ( $10^6$  -  $10^8$  organisms), *Salmonella* spp. (10 s), *Shigella* spp. (10 s), *Vibrio* spp. ( $10^4$  -  $10^6$  organisms). However, *Vibrio* spp. can cause disease with a minimum of 106 cells in some rare case [13].

The aim of this study was to isolate various type of gram negative bacteria from Street sauce samples collected from various locations of Dhaka city and compare it with Brand Sauces to observe whether the bacterial count of those samples are below or above the minimum infective dose to evaluate their safety margin.

## 2. Materials and Methods

### 2.1 Collection of Samples

Nine different sauce samples were collected from different the areas of Dhaka city. Four of those samples were brand sauce Ahmed, Pran, Ruchi and Radhuni and the others were local street sauce collected from Mohammadpur, Dhanmondi, Mirpur, Banani and Gulshan area.

### 2.2 Selective Media

Four types of selective media were used

1. Mac-Conkey Agar (MAC)
2. Thiosulfate-Citrate-Bile Salts-Sucrose Agar (TCBS agar)
3. Salmonella- Shigella Agar (SS agar)
4. Eosin-Methylene Blue (EMB)

### 2.3 Spread Plate Method

Spread plate technique is the method of isolation and enumeration of microorganisms in a mixed culture and distributing it evenly. The technique makes it easier to quantify bacteria in a solution. In this technique, the sample is appropriately diluted and a small aliquot transferred to an agar plate. The bacteria are then distributed evenly over the surface by a sterilized spreader. [14].

### 2.4 Serial Dilution

At first serial dilution of each sauce sample was performed by adding 1 ml of raw sauce sample with 9 ml of dilution media (1:10 dilution). After that it was continued in 1:100, 1:1000, 1:10000 and 1:100000 ratios. Sterile water was used as dilution medium. After that samples were cultured on

selective media plates by spread plate techniques and incubated at 37 °C for 24 hours for bacterial growth. After that the colony forming units on each selective media plates were counted.

### 2.5 Biochemical tests

Different types of gram negative bacteria were identified by observing their color in the selective media and then by performing biochemical tests upon similar colonies.

### 2.6 Catalase

To find out if the particular bacterial isolate is able to produce catalase enzyme, small inoculum of bacterial isolate is mixed into hydrogen peroxide solution (3%) and the rapid elaboration of oxygen bubbles occurs. The lack of catalase is evident by a lack of or weak bubble production. It was done by picking a pure colony by a sterile loop and immersing it in 2 drops of 3% H<sub>2</sub>O<sub>2</sub> solution in a glass slide. Production of bubbles indicated the positive results [15].

### 2.7 Oxidase

The test was done to detect the presence of cytochrome oxidase in the organism. A single colony was picked up with a sterile toothpick and rubbed on to whatman filter paper that is soaked with 2-3 drops of N, N, N', N'-tetramethyl-p-phenylenediamine dihydrochloride. Positive result was recognized by a dark purple color within 5-10 seconds [16].

### 2.8 Motility Indole Urea (MIU)

One isolated colony was touched with a sterile wire and stabbed into semisolid agar medium very carefully down the tube, without touching the bottom. The tube was incubated at 37 °C for 18 to 24 hours. Non-motile bacteria generally give growths that are confined to the stab-line, have sharply defined margins and leave the surrounding medium clearly transparent. Motile Bacteria typically give diffuse, hazy growths that spread throughout the medium rendering it slightly opaque [17].

### 2.9 Citrate Utilization test

Simmons citrate agar tests the ability of organisms to utilize citrate as a carbon source. Simmons citrate agar contains sodium citrate as the sole source of carbon, ammonium dihydrogen phosphate as the sole source of nitrogen and the pH indicator bromothymol blue. If the medium turns blue, the organism is citrate positive. If there is no color change, the organism is citrate negative [18].

### 2.10 Methyl Red test (MR)

An isolate is inoculated into a tube with a sterile transfer loop. The tube is incubated at 35 °C (95 °F) for 2–5 days. After incubation, 2.5 ml of the medium are transferred to another tube. Five drops of the pH indicator methyl red is added to this tube. The tube is gently rolled between the palms to disperse the methyl red. Enterics that subsequently metabolize pyruvic acid to other acids lower the pH of the medium to 4.2. At this pH, methyl red turns red, indicating a positive test. Enterics that subsequently metabolize pyruvic acid to neutral end products lower the pH of the medium to only 6.0. At this pH, methyl red is yellow, indicating a negative test [19].

### 2.11 Lactose fermentation test

An inoculum from a pure culture is transferred aseptically to a sterile tube of phenol red lactose broth. The inoculated tube is

incubated at 35-37 C for 24 hours and the results are determined. A positive test consists of a color change from red to yellow, indicating a pH change to acidic [20].

### 3. Results and Discussion

#### 3.1 Isolation of Bacteria from Samples

**Table 1:** Colony forming units in different selective media for Brand sauce (AHMED)

Dilution	MAC	TCBS	SS	EMB
Raw	9	0	0	10
10 <sup>-1</sup>	7	0	0	8
10 <sup>-2</sup>	4	0	0	4
10 <sup>-3</sup>	1	0	0	1
10 <sup>-4</sup>	0	0	0	0
10 <sup>-5</sup>	0	0	0	0

**Table 2:** Colony forming units in different selective media for Brand sauce (PRAN)

Dilution	MAC	TCBS	SS	EMB
Raw	13	0	0	28
10 <sup>-1</sup>	10	0	0	19
10 <sup>-2</sup>	7	0	0	15
10 <sup>-3</sup>	4	0	0	9
10 <sup>-4</sup>	2	0	0	5
10 <sup>-5</sup>	1	0	0	2

**Table 3:** Colony forming units in different selective media for Brand sauce (RUCHI)

Dilution	MAC	TCBS	SS	EMB
Raw	5	1	0	11
10 <sup>-1</sup>	2	0	0	5
10 <sup>-2</sup>	0	0	0	1
10 <sup>-3</sup>	0	0	0	0
10 <sup>-4</sup>	0	0	0	0
10 <sup>-5</sup>	0	0	0	0

**Table 4:** Colony forming units in different selective media for Brand sauce (RADHUNI)

Dilution	MAC	TCBS	SS	EMB
Raw	14	0	0	11
10 <sup>-1</sup>	9	0	0	6
10 <sup>-2</sup>	2	0	0	1
10 <sup>-3</sup>	0	0	0	0
10 <sup>-4</sup>	0	0	0	0
10 <sup>-5</sup>	0	0	0	0

**Table 5:** Colony forming units in different selective media for Street sauce (Mohammadpur)

Dilution	MAC	TCBS	SS	EMB
Raw	82	95	5	79
10 <sup>-1</sup>	53	71	1	57
10 <sup>-2</sup>	40	54	0	32
10 <sup>-3</sup>	26	33	0	17
10 <sup>-4</sup>	14	14	0	6
10 <sup>-5</sup>	4	5	0	1

**Table 6:** Colony forming units in different selective media for Street sauce (Dhanmondi)

Dilution	MAC	TCBS	SS	EMB
Raw	104	84	11	124
10 <sup>-1</sup>	88	52	4	95
10 <sup>-2</sup>	63	36	1	66
10 <sup>-3</sup>	39	15	0	42
10 <sup>-4</sup>	18	9	0	26
10 <sup>-5</sup>	3	2	0	8

**Table 7:** Colony forming units in different selective media for Street sauce (Mirpur)

Dilution	MAC	TCBS	SS	EMB
Raw	114	108	16	132
10 <sup>-1</sup>	78	77	5	96
10 <sup>-2</sup>	40	32	1	63
10 <sup>-3</sup>	22	12	0	37
10 <sup>-4</sup>	10	4	0	20
10 <sup>-5</sup>	1	0	0	5

**Table 8:** Colony forming units in different selective media for Street sauce (Gulshan)

Dilution	MAC	TCBS	SS	EMB
Raw	52	4	1	71
10 <sup>-1</sup>	31	0	0	44
10 <sup>-2</sup>	19	0	0	28
10 <sup>-3</sup>	7	0	0	12
10 <sup>-4</sup>	3	0	0	4
10 <sup>-5</sup>	0	0	0	0

**Table 9:** Colony forming units in different selective media for Street sauce (Banani)

Dilution	MAC	TCBS	SS	EMB
Raw	92	27	15	155
10 <sup>-1</sup>	71	16	9	117
10 <sup>-2</sup>	53	9	4	82
10 <sup>-3</sup>	34	3	1	53
10 <sup>-4</sup>	13	0	0	28
10 <sup>-5</sup>	4	0	0	6

#### 3.2 Biochemical test results

**Table 10:** Identification of colony forming units isolated in MAC agar media

Color	Catalase	Oxidase	MR	MIU	Citrate Utilization	Lactose	Identification
Pink	+	-	+	M + I + U -	-	+	<i>Escherichia coli</i>
White	+	-	+	M + I - U -	-	-	<i>Salmonella typhi</i>
Colorless	-	-	+	M - I + U -	-	-	<i>Shigella dysenteriae</i>

**Table 11:** Identification of colony forming units isolated in EMB agar media

Color	Catalase	Oxidase	MR	MIU	Citrate Utilization	Lactose	Identification
Metallic green	+	-	+	M+ I+ U-	-	+	<i>Escherichia coli</i>
White	+	-	+	M+ I- U-	-	-	<i>Salmonella typhi</i>
Colorless	-	-	+	M- I+ U-	-	-	<i>Shigella dysenteriae</i>

**Table 12:** Identification of colony forming units isolated in SS agar media

Color	Catalase	Oxidase	MR	MIU	Citrate Utilization	Lactose	Identification
Colorless with black center	+	-	+	M+ I- U-	-	-	<i>Salmonella typhi</i>
Colorless	-	-	+	M- I+ U-	-	-	<i>Shigella dysenteriae</i>

**Table 13:** Identification of colony forming units isolated in TCBS agar media

Color	Catalase	Oxidase	MR	MIU	Citrate Utilization	Lactose	Identification
Yellow	+	+	-	M+ I+ U-	+	-	<i>Vibrio cholera</i>
Bluish green center	+	+	+	M+ I+ U-	-	-	<i>Vibrio parahaemolyticus</i>

#### 4. Discussion

Biochemical tests displayed presence of *Escherichia coli*, *Salmonella typhi*, *Shigella dysenteriae*, *Vibrio cholera* and *Vibrio parahaemolyticus*. The results displayed above showed that all the Brand Sauce samples contained some amount of bacterial colony forming units. Almost all the colony forming units isolated from Brand sauce samples in EMB media displayed distinctive metallic green color which indicates presence of lactose fermenting organisms such as *Escherichia coli*. However, minimum infective dose of these organisms are quite high ( $10^6$  -  $10^8$  organisms). The colony forming units isolated in EMB media which did not display metallic green color indicates the presence of non-lactose fermenting organisms such as *Salmonella* spp. and *Shigella* spp. The minimum infective dose of such organism are very low (10 s) but the amount found in EMB of all Brand sauce samples were a lot lower than that.

All the Brand sauce samples isolated in the SS agar media displayed no colony forming units. Three of the four Brand sauce samples displayed no colony forming units in the TCBS agar media only one of them displayed a single colony forming unit.

Almost all of the Brand sauce samples isolated in the MAC agar displayed pink colony forming units which indicates lactose fermenting organisms. Some colony forming units were colorless indicating non-lactose fermenting organism. Amount of both these type of colony forming units were a lot lower than minimum infective dose.

All the Street Sauce samples isolated in the MAC and EMB media displayed more colony forming units comparing with Brand sauce samples. However, amount of lactose fermenting colonies were lower than minimum infective dose. The non-lactose fermenting colonies in the EMB and MAC media isolated from most of the Street sauce samples were found to be more than minimum infective dose. All of the Street sauce

samples isolated in the SS media displayed colony forming units. Three of the five Street sauce samples displayed more than 10 colony forming units in the SS media which is more than the minimum infective dose of both *Salmonella* spp. and *Shigella* spp.

All Street Sauce samples isolated in the TCBS media displayed some amount of colony forming units. Most of these were yellow indicating *Vibrio cholera* and some of them had blue to green centers indicating *Vibrio parahaemolyticus*. The usual infection dose of *Vibrio* spp. is quite large ( $10^4$ - $10^6$  organisms). However, on many cases minimum infective dose was found to be 106 colony forming units. Two of the five Street sauce samples displayed colonies near the minimum infection dose and another one was above 106.

All the Street sauce samples isolated in different selective media displayed a huge amount of colony forming units comparing with the Brand sauce samples clearly differentiating between the industrial manufacturing process and high quality of preservatives used in them from the normal manufacturing process of local manufacturers. This clearly shows that the Brand Sauce manufacturers maintain the safety margin a lot more than Street Sauce manufacturers.

#### 5. Conclusion

The above study showed that microbial loads in Brand products are less than local products. The brand products are safer for human consumption. Most of the street sauces are seriously counted with pathogenic gram negative bacteria. Brand sauces like Ahmed, Pran, Ruchi and Radhuni are far better than street sauce however further studies should be carried out to confirm their safety margin more accurately.

#### 6. Conflict of Interest

There is no conflict of interest between the authors or any institutions about this article.

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