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Assessing prevalence of antibiotic resistant microbes on fresh marketed vegetables of Aizawl city

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ABSTRACT

Aims: The study was carried out to evaluate the isolation and identification of the prevalent bacterial flora and their antibiotic resistance pattern from fresh vegetables sold in the local markets of Aizawl town.
Methodology and results: Three vegetables:- Tomato (*Lycopersicon esculentum*), potato (*Solanum tuberosum*) and cabbage (*Brassica Oleracea*) were randomly collected from different vendors of three local markets in Aizawl town. All the vegetables were washed with double distilled water and cultured onto Mac-Conkey agar and sub-cultured into nutrient agar to obtain pure culture for the identification. Samples were analyzed to study the density of microorganisms by standard plate count (SPC). Mean microbial load ranged from 2.46×10^5–11.85×10^5 CFU/mL for market A; 1.3×10^4–2.51×10^6 CFU/mL for market B and 1.09×10^5–3.14×10^6 CFU/mL for market C. Approximately 41 bacterial isolates made up of 7 genera of bacteria were made from the 3 vegetable groups. *Enterobacter* spp. (39.02%), *Klebsiella* spp. (26.82), *Proteus* spp. (9.76%), *Staphylococcus* spp. (9.76%) and *E. coli* (4.88%), Citrobacter sp. (4.88%) and *Serratia* spp. (4.88%) were the bacteria species isolated. Antibiotic sensitivity patterns of the isolates were determined and almost all of them were resistant to commonly used antibiotics. The percentage of Multi Drug Resistant (MDR) bacteria against the total load was very high (80.5%), and the ESBL production is 62.16%.
Conclusion, significance and impact study: Vegetable contamination with bacteria was observed in all the three markets A, B and C. Raw vegetables from the markets were considered unfit for making fresh produce for human consumption and adequate cooking with proper handling before consumption is suggested.

Keywords: Aizawl, Antibiotic resistance, vegetable pathogens, vegetables quality.

INTRODUCTION

Fresh vegetables are essential parts of the diet of humans. Fruits and vegetables are rich in nutrients, micronutrients and vitamins which help the consumers from vitamin deficiencies, malnutrition and to get rid of the diseases related to these. Fruits and vegetables contain good amount of vitamins mainly vitamin C and A, which are required for the treatment of most of the infections as a supplement along with the medicines (Kalia and Gupta, 2006).

Fruits and vegetables are exposed to microbial contaminations that are mainly due to the high nutrition, neutral pH, micro nutrition and other favorable conditions required for the microbial growth. Vegetables harbor both human and plant microbes since they are widely exposed to the sewage contaminated soil, water and manures (Nguyen-the and Carlin, 1994; Carmo *et al*., 2004). These vegetables can also get contaminated with pathogenic microbes during harvesting, transportation, storage, transport containers, retailer handling etc. Due to these wide range of exposure to different source microbial flora of the vegetables greatly vary (Ray and Bhunia, 2007; Ofor *et al*., 2009).

Most microorganisms that are initially observed on whole fruit or vegetable surfaces are soil inhabitants which have members of a very large and diverse community of microbes. Transmission of microbes to these vegetables may also occur through vectors like soil particles, airborne spores and irrigation water. Microbes that harbor on the vegetable surface may cause spoilage due to favorable conditions found for their growth. Although spoilage microbes including bacteria, yeasts and moulds dominate, human pathogens like pathogenic bacteria, viruses and parasites occasionally present on vegetables are also documented (Hasan *et al*., 2006).

An increased number of microbial infections associated with consumption of fresh vegetables have been reported in recent years. Documented illnesses have been caused by bacteria, parasites, and viruses and are

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transmitted via many types of fruits and vegetables (Beuchat, 1996; Nyenje *et al*., 2012). Excessive and misuse of antimicrobials to control pathogens in animals and crops led to antibiotic resistance and transfer to human through contaminated food. Some researches show that antibiotic resistant bacteria also may be ingested with vegetables (Kilonzo *et al*., 2009). Vegetables such as corn, green onion and cabbage absorb antibiotics when grown in soil fertilized with livestock antibiotics contaminated manure (Kumar *et al.*, 2005).

Antibiotic resistance is a major problem in agriculture, livestock and medical field for many reasons (CDC, 2005). First, antibiotic resistance found against the penicillin group of drugs, now it is increasing to antibiotics of fluoroquinolones and third generation cephalosporins. These are commonly used antibiotics to treat serious infections of bacteria in humans like *Salmonella* and *Shigella* species etc.

Consumption of vegetables and fruits mainly uncooked or unprocessed represents the direct human exposure to spoilage microbes found on them. In our current study, we assessed the prevalence of antibiotic resistant pathogens on fresh vegetables like potatoes, tomatoes and cabbage collected from markets.

MATERIALS AND METHODS

Collection of sample

During the study period i.e. from September, 2013 to February, 2014 a total of 27 samples (visits) of tomato, cabbage and potato were collected in sterile polythene bags from 3 different major vegetable markets (Zemabawk, Thuampui and Bara bazaar) of Aizawl town, Mizoram, North East India. All the samples were collected after at least one week gap, so that there is no repeated sampling from the same stock. All the samples in this study were freshly collected and hygienically packed transported to the microbiology laboratory, Department of Medical Laboratory Technology (MLT) of Regional Institute of Paramedical and Nursing sciences (RIPANS), Aizawl. The samples were processed immediately after collection; it was refrigerated at 2 °C to 8 °C if processing was delayed more than one day.

Isolation, enumeration and identification of bacteria by Standard Plate Count (SPC) method

All the vegetable samples were rinsed thoroughly in a 250 mL beaker containing 100 mL of sterile distilled water. Then rinsed water samples or surface cleaned samples were serially diluted up to 10^{-7}. A 0.1 mL of each dilution was spread on Mac-Conkey agar (HiMedia Pvt. Ltd. Mumbai, India) and the plates were incubated at 37 °C for 24 h for isolation of bacteria (Khan *et al*., 1992). Total viable counts were determined by counting both red and non-red colonies on the Mac-Conkey plates. Based on the morphological characteristics, red and non-red colonies were selected from Mac-Conkey agar plate and sub cultured to Nutrient agar for identification by all the standard bacteriological methods viz. Gram staining, biochemical tests namely: IMViC (Indole, Methyl Red, Voges-Proskauer, citrate), triple sugar iron; and sub-culture on differential media (Eosin Methylene Blue (EMB) agar at 37 °C and 44 °C for 24 to 48 h; Mannitol salt agar and in Cystein Lactose Electrolyte Deficient (CLED) agar at 37 °C) and also in nutrient agar (Holt *et al*., 1994).

Gram staining

A loop full of overnight culture was placed on the slide with a drop of distilled water. Smear was prepared by spreading the drop of inoculum with inoculation loop. The heat fixed smear was first stained with crystal violet for 60 sec. After rinse the slide, it was flooded with Grams iodine solution and was kept for 60 sec. Slide was again washed under the tap water and added 95% alcohol for 30 sec. After wash the slide, it was stained with saffranin for 60 s. It was again rinsed under tap water and dried on paper towels. The cells were examined under the light microscope.

Biochemical tests

Biochemical tests were done according to Collee's Mackie & McCartney Practical Medical Microbiology (Collee *et al*., 2011). Biochemical tests conducted in this study were as follows: Catalase Test, Triple Sugar Iron Agar (TSI) test, Hydrogen sulfide production (H_2S), Methyl Red (MR) test, Voges-Proskauer (VP) test, Citrate Utilization test, Nitrate reduction test, Indole production and Motility tests were performed to identify the bacteria up to the Genus level.

Motility determination

A small amount of Vaseline was placed at each corner of clean cover glass. Two loopful of the 24 h old culture of the organism from nutrient broth was placed at the center of the cover glass. A concavity slide was pressed over the cover glass, such that the depressions cover the culture drop and quickly inverted. The completed preparation was observed microscopically.

Antibiotic susceptibility tests

Briefly, the susceptibility of all the isolates against the antimicrobials was determined by Kirby-Bauer disc diffusion method in Mueller-Hinton agar (Bauer *et al.,* 1966). The inoculum was prepared at a density adjusted to a 0.5 McFarland turbidity standard solution. Commercially available antimicrobial discs (HiMedia Ltd, Mumbai, India) of Aztreonam (AT10 = 10 µg), Nitrofurantoin (NIT200 = 200 µg), Ciprofloxacin (CF10 = 10 µg), Ampicillin (A10 = 10 µg), Ceftazidime (CFM5 = 5 µg), Meropenem (MRP10 = 10 µg), Imepenem (IPM10 = 10 µg), Linezolid (LZ30 = 30 µg), Cefoxitin (CX30 = 30 µg), Co-timoxazole (CO30 = Trimethoprim 2.50 µg and Sulphamethoxazole 27.5 µg), Norfloxacin (NX10 = 10 µg),

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Table 1: Showing the microbial load on chosen vegetables from Aizawl Markets.

Sl. No.	Name of isolates	Number of occurrence	Percentage (%)
1.	Escherichia coli	2	4.88
2.	*Enterobacter* sp.	16	39.02
3.	*Klebsiella* sp.	11	26.82
4.	*Proteus* sp.	4	09.76
5.	*Citrobacter* sp.	2	04.88
6.	*Staphylococcus* sp.	4	09.76
7.	*Serratia* sp.	2	04.88
Total		41	100 %

Cephotaxime (CE30 = 30 µg), Amikacin (AK10 = 10 µg) and Clindamycin (CD10 = 10 µg) were placed on the inoculated agar plates and incubated in an upright position overnight at 37 °C. Sensitivity was recorded after 24 h of incubation by measuring the zone of inhibition formed around the antimicrobial discs. The results were expressed as Sensitive, Intermediate and Resistant by considering CLSI, 2012 guidelines.

RESULTS

Bacteria can cause fruits and vegetables to get mushy, slimy and make them produce bad odour. There are different spoilage bacteria which grow well at room temperature. The large number of microorganism's and their waste products causes the objectionable changes in odour, taste and texture. Antibiotic resistance is a worldwide problem in the medical society that continues to grow. It is again a major concern in public health that risk of consuming vegetables and fruits harboring drug resistant bacteria. Hence it is necessary to screen the vegetable, fruits and food items which we consume for the energy every day, since there is an enormous chance of getting food pathogens than from the other sources. For the current study, we have collected samples from three different local markets of Aizawl city, Mizoram, North eastern state in India.

Total Viable Count (TVC)

The microbial load of the samples varied with type and vendors or markets (Table 1). Range of microbial count was varied to different markets, it ranged from 2.46×10^5 to 11.85×10^5 CFU/mL for Zemabawk market (A), 1.3×10^4 to 5.92×10^5 CFU/mL for Thuampui market (B) and 1.09×10^5 to 3.14×10^6 CFU/mL for Bara bazaar market (C). Tomato from the market B had the lowest microbial load (1.09×10^4 CFU/mL) of all the samples collected while cabbage from market C had the highest microbial load (3.14×10^6 CFU/mL). Among all the tomato samples least microbial load was found from market B (1.3×10^4 CFU/mL) and highest from market A (11.85×10^5 CFU/mL), potato from market A had the least microbial count (2.46×10^5 CFU/mL) and highest from the market C (1.59×10^6 CFU/mL). Cabbage had the least microbial count from market A (3.57×10^5 CFU/mL) and highest from the market C (3.14×10^6 CFU/mL). Mean TVC (log CFU/mL) and Standard deviation, Standard error of mean etc. were calculated statistically to find the significant difference in the microbial load of LF and NLF from the collected vegetables. There were significant differences found between LF and the NLF isolates. ($p \leq 0.05$) (Table 2).

Percentage of isolates occurrence

Bacterial isolates were isolated and identified upto the Genus level by sub culturing to MacConkey agar, Cystein Lactose Electrolyte Deficient (CLED) agar and Mannitol Salt Agar (MSA) and incubated at 37 °C for 24 h. By standard bacteriological tests viz. Gram staining, catalase test, coagulase test, IMViC, Triple Sugar Iron Agar (TSI) tests, Motility test and Eosin Methylene Blue (EMB) agar were used to identify to the genus level.

Table 2: Showing the percentage of the isolates from all the vegetable samples.

Sampling sites	Microbial load (CFU/mL)					
	Tomato		Potato		Cabbage	
	LF	NLF	LF	NLF	LF	NLF
Zemabawk (A)	11.85×10^5	7.12×10^5	3.67×10^5	2.46×10^5	8.22×10^5	3.57×10^5
Thuampui (B)	5.92×10^5	1.3×10^4	3.46×10^5	3.45×10^5	2.51×10^6	1.22×10^6
Bara Bazaar (C)	1.39×10^6	1.09×10^5	1.59×10^6	1.30×10^6	3.14×10^6	1.94×10^6
SD values	4.1×10^5	3.7×10^5	7.1×10^5	5.8×10^5	1.2×10^5	7.9×10^5
p values	0.0459		0.1201		0.0319	

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Table 3: Showing the isolates Multi Drug Resistance patterns against the selected antibiotics.

Sl. no.	Organism (No. of isolates)	AT 30	NIT 300	CF 10	A 10	CFM 5	CAZ 30	MRP 10	IPM 10	LZ 30	CX 30	CO 25	NX 10	CE 30	AK 10	CD 10	MDR (%)
1.	*Escherichia coli* (n =2)	1 (50)	0	1 (50)	2 (100)	1 (50)	1 (50)	0	0	0	1 (50)	2 (100)	1 (50)	1 (50)	0	1 (50)	1 (50)
2.	*Enterobacter* sp. (n =16)	6 (37.5)	12 (75)	6 (37.5)	10 (62.5)	9 (56.25)	9 (56.25)	4 (25)	6 (37.5)	9 (56.25)	10 (62.5)	6 (37.5)	4 (25)	9 (56.25)	5 (31.5)	7 (43.75)	12 (75)
3.	*Klebsiella* sp. (n =11)	9 (81.81)	10 (90.90)	2 (18.18)	10 (90.90)	10 (90.90)	5 (45.45)	1 (9.09)	1 (9.09)	9 (81.81)	7 (63.63)	9 (81.81)	1 (9.09)	9 (81.81)	4 (36.36)	10 (90.90)	10 (90.90)
4.	*Proteus* sp. (n =4)	2 (50)	3 (75)	2 (50)	3 (75)	2 (50)	2 (50)	0	0	2 (50)	1 (25)	3 (75)	1 (25)	2 (50)	1 (25)	3 (75)	3 (75)
5.	*Citrobacter* sp. (n=2)	0	0	0	2 (100)	1 (50)	0	0	0	1 (50)	0	2 (100)	0	2 (100)	0	1 (50)	1 (50)
6.	*Staphylococcus* sp. (n=4)	3 (75)	2 (50)	2 (50)	4 (100)	2 (50)	2 (50)	0	0	2 (50)	2 (50)	2 (50)	3 (75)	2 (50)	1 (25)	1 (25)	3 (75)
7.	*Serratia* sp. (n=2)	0	0	1 (50)	1 (50)	0	0	0	0	1 (50)	1 (50)	1 (50)	0	0	0	0	0
Total (% resistance)	(n = 41)	21 (51.21)	27 (65.85)	14 (34.14)	32 (78.04)	25 (60.97)	19 (46.34)	5 (12.19)	7 (17.07)	24 (58.53)	22 (53.65)	25 (60.97)	10 (24.39)	25 (60.97)	11 (26.82)	23 (56.09)	30 (73.17)

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A total of 41 isolates was isolated from all the types of vegetables collected. Isolated bacterial isolates were almost all Gram negative with only 5% of contribution made by Gram positive cocci i.e. *Staphylococcus* spp. of the total isolates. Physiological and biochemical tests showed that the isolated organisms comprised of seven genera viz. *Enterobacter* spp. (39.02%), *Klebsiella* spp. (26.82%), *Escherichia coli* (4.88%), *Citrobacter* spp. (4.88 %) and *Serratia* spp. (4.88 %) as Lactose Fomenters (LF) and *Proteus* spp. (9.76%) as Non-Lactose Fermenter (NLF) and *Staphylococcus* spp. (9.76%)of Gram positive cocci (Table 2).

Antimicrobial resistance

An antibiogram study of 41 isolates showed that the percentage of resistance was very high. Among the 41 isolates tested for drug resistance the higher percentage of resistance was found against Ampicillin (78.04%), followed by 65.85% resistance to Nitrofurantoin and 60.97% against three antibiotics that Ceftazidime, Cephotaxime and Co-Timoxazole (Table 3). The antibiotics to which maximum isolates found sensitive were against Meropenem, Imepenem, Norfloxacin and Amikacin and only 12.19%, 17.07%, 24.39% and 26.82% isolates were found resistant to these antibiotics respectively. These experimental results also suggested that multi drug resistance of environmental bacteria is increasing and almost all the isolates were resistant to more than five antibiotics.

In this antibiogram study 15 antibiotics were used and any bacteria shows resistance to more than 7 antibiotics was considered to be Multi Drug Resistant (MDR) isolates. Only two isolated genus were showing resistance to all the antibiotics used i.e. *Enterobacter* spp. and *Klebsiella* spp. and the rest of the species contribute some percentage to Non-MDR isolates. The MDR analysis has shown that 85.37% of the isolates are MDR and only 14.63% are Non-MDR isolates

Extended spectrum of β-lactamases

All the Gram negative isolates have been tested for the Extended spectrum β-lactamase enzymes (ESBL) by standard bacteriological double diffusion synergy test (DDST) methods (CLSI, 2012). Of all the members of Enterobacteriaceae Gram Negative Bacilli (GNB) isolates *Klebsiella* spp. was found to be the highest ESBL producer that 29.73%, followed by *Enterobacter* spp. (24.32%). *Proteus* spp. (5.40%) and *E. coli* (2.70%) contributes very less percentage of ESBL productions, but 0% of isolates found positive for ESBL from the genus *Citrobacter* spp. and *Serratia* spp. (Table 4).

DISCUSSION

Freshly consumed vegetables especially those use in salad mixtures, have been implicated in food poisoning and thus hazardous to the health of the consumers. This could be linked to the fact that most of these vegetables are consumed without being subjected to the thermal process or even thorough washing (Lund, 1992). The microbial load present in fruits and vegetables are the direct reflection of the sanitary quality of the cultivation water, harvesting, transportation, storage and processing of the produce (Beuchet, 1996). All the bacteria isolated in this study have previously been isolated from fruits and vegetable in other studies in India and elsewhere (Khan *et al*., 1992; Mosupuye and Holy, 1999; Viswanathan and Kaur, 2001; Sivapalasingam *et al*., 2004; Tambedkar and Mundhada, 2006; Ankita Rajvanshi, 2010).

Table 4: Showing the percentage of prevalent ESBL producing Enterobactericeae on vegetables of Aizawl markets.

Sl. No.	Organisms (Enterobacteriaceae members)	Percentage of isolation	Percentage of ESBL
1.	*Escherichia coli*	2 (5.40%)	1 (2.70%)
2.	*Enterobacter* sp.	16 (43.24%)	9 (24.32%)
3.	*Klebsiella* sp.	11 (29.73%)	11 (29.73%)
4.	*Proteus* sp.	4 (10.81%)	2 (5.40%)
5.	*Citrobacter* sp.	2 (5.40%)	0
6.	*Serratia* sp.	2 (5.40%)	0
Total (n = 37)		37 (100%)	23 (62.16%)
SD values		16.074	13.176
SEM		6.562	5.329
p value (one tail)		0.0324	

The higher microbial load observed in the fruits and vegetables in this current study may be a reflection of storage conditions and how long these produce were kept before they were obtained for sampling. Bacteria on storage may get transferred from one to another vegetable during pre-washing by some contaminated water, kept in a bag used for long time storage where bacteria may multiply over time depending on the storage conditions especially those are psychotropic and mesophilic (Montville and Mathews, 2008). The high bacteria counts observed from fruits and vegetables in this current study are similar to those obtained in other studies in India (Viswanathan and Kaur, 2001).

The presence of *S. aureus*, a pathogenic organism of public health concern, in most of the samples and the presence of other pathogenic and opportunistic bacteria like *E. coli*, *Proteus* spp. and *Klebsiella* spp. in some of the fruits and vegetables, further highlights the need to safeguard the health of the consumers by proper washing and decontamination of these produce which are consumed without heat treatment.

Enterobacter spp. (39.02%) was the most common isolate in all the three sample groups of the current study. These findings are analogous to the reports from Finland and Nigeria. A higher rate of *Enterobacter* spp. prevalence was reported in Finland and showed that 38.3% of all the three samples used i.e. Fresh Finnish, Fresh imported and Frozen imported (Monica *et al*., 1999). *Enterobacter*

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aerogenes were also found in higher percentage (56%) of prevalence in vegetable salads sold at restaurants of Okada town, Edo State, Nigeria (Osamwonyi *et al.*, 2013).

Mosupuye and Von Holy (1999) reported the presence of *Salmonella* in street foods in South Africa. Viswanathan and Kaur (2001) showed the presence of *Salmonella*, *Serratia*, *Enterobacter*, *Staphylococcus aureus*, faecal *E. coli* and *P. aeruginosa* in vegetables and fruits. The present study also showed the presence of *E. coli*, *Enterobacter*, *Serratia* and *Staphylococcus* in fresh vegetables collected from three different vendors. WHO (2002) reported that *Salmonella* spp. causes Salmonellosis and typhoid fever and *E. coli* O157:H7 causes severe illness and deaths, especially among children in several countries. Although the species distribution in our material, with very few *E. coli*, the dominant aerobic rod in fecal flora, suggests that fecal contamination rate is rare.

Antimicrobial agents are used widely as food additives to improve growth and feed conversion in many types of animal operations, including poultry, swine and cattle operations. And in humans misuse of antibiotics or self medication is so common. As a result, antibiotic resistance in the bacterial communities in the intestinal tracts of domestic animals and in humans has become common (Aerestrup *et al*., 2000). The emergence of drug resistance is one of the most serious health problems in developing countries like India. In this study, the high antibiotic resistance rate (80.5%), numerous resistance pattern and high ESBL producing pathogens (62.16%) were found prevalent in the vegetables. Thus the material in this study gives a more reliable picture of the resistance levels that can be expected in most Enterobacteriaceae on vegetable in North East India.

Multi drug resistance (MDR) pattern was observed in almost all the strains isolated. Among the 41 strains isolated 33 (80.5%) were MDR strains which are very high compared to previous report by the author in UTI patients from the same study area (Karuppasamy and Lalsanglura, 2012). Of those, except *Serratia* spp. all other strains were found resistant to more than 5 drugs at least. Drug resistance percentage is increased in comparison to the previous study, Nitrofurantoin 27.16% increased to 65.85 %, Amikacin 7.41% increased to 26.82% and 0% strains were resistant to Meropenem and Imepenem which is now showing 12.19% and 17.01% respectively in this study.

ESBLs

Extended Spectrum β Lactamases (ESBLs) are a group of enzymes that have the common property of providing resistance to extended-spectrum β lactam antibiotics such as Oxyimino cephalosporins (e.g. cefotaxime, ceftazidime, ceftriaxone, cefepime and cefpirome), as well to aztreonam an oxyimino monobactam, Cephamycins (Cefoxitin and cefotetan) and Carbapenems (Imepenem and Meropenem) (Oreste, 2003). In this study 23 (62.16%) strains out of 37 GNB found to be positive for the ESBLs double disk synergy test (DDST) (CLSI, 2012). This indicates that the resistance genes might have transferred to the pathogens of vegetables from human pathogens or vice versa. This high prevalence of ESBLs resistance bacteria on the fresh vegetables is representing a high potential health risk to human population consumes or exposed to these vegetables of the current study locations.

CONCLUSION

To limit the introduction of pathogenic bacteria to vegetables through irrigation, the origin and distribution of irrigation water should be known. Where there are wells used, wells should be well-maintained, and all irrigation sources should be monitored routinely for human pathogens (Buck *et al*., 2003). Manure used as fertilizer should be treated either by composting or aging to eliminate pathogenic microorganisms and farmers should be educated about the manure application and harvest. Fruits and vegetables processor should be educated on the adverse effect of using untreated or polluted water for contamination. Processors/vendors should also observe strict hygienic measures to ensure that they do not serve as a source of chance for inoculation of microorganisms/contamination. There is need to make a law compelling vendor, in Aizawl, to transport/sell fresh fruits and vegetables in cool temperature controlled carts similar to those used for the transport /sales of yogurt and ice creams.

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