Microbiological Study of Food Packaging Paper of Kathmandu Valley

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ABSTRACT

Objectives: The purpose of this study was to isolate and identify microorganisms of food packaging papers of Kathmandu valley and determine antibiotic susceptibility of the isolates.

Methods: A total of 34 food packaging paper samples were collected aseptically from hotels, bakeries and sweet shops (considered as closed shop) and open street vendors and were transported to microbiology laboratory of Golden Gate International College for processing. The isolates were identified by standard microbiological procedures and subjected to antimicrobial susceptibility testing by modified Kirby-Bauer disk diffusion method following CLSI guidelines. The rate of Extended Spectrum Beta- lactamase (ESBL) producing and multiple drug resistant (MDR) isolates were also determined.

Results: All 34 samples yielded microbial growth with average microbial count of 4.145×10^5 CFU/g. Among 103 microbial isolates, 78 were bacteria, 15 molds and 10 yeasts. The predominant bacterial and mold isolates were *Bacillus* spp (43.59%) and *Cladosporium* spp (46.67%) respectively. Ciprofloxacin (42/43) and Amikacin (42/43) were the most effective and ampicillin (39/43) was most resistant antibiotics for Gram negative bacteria. A total of 9.30% Gram negative isolates were identified as ESBL producing and MDR strains.

Conclusion: This result indicates that potential pathogens are found in food packaging papers which can be threat to health of consumers as they may act as a source of food borne infection.

Keywords: Food packaging papers, antibiotic susceptibility testing, MDR, ESBL

INTRODUCTION

Enormous number of people consume several varieties of foods which are generally served in recycled papers such as abandoned recycled newspapers (Hladikova et al. 2015). The main ingredient of all paper is biodegradable plant material cellulose fibers, hemicellulose and lignin. Besides, loading or filling materials like $CaCO_3$, Talc and other several other chemicals depending on the type of paper may be used (Guzińska et al. 2012).

The biodegradable constituents can enhance microbial growth in paper and paperboard packaging whereas contamination can occur a result of contaminated raw materials used in paper production, during processing of raw materials, during transportation and during handling (Mohammadzadeh-Vazifeh et al. 2015).

As a packaging material, newspapers, academic papers, hospital report papers are also used. These papers often come in direct contact with food like Samosa, Chatpate, Paratha, pakoda, bakery products and other Nepali street foods. These re-used papers may be already contaminated when stored in dirty and damp places (Rana et al. 2019). The contaminating microbes can decay food (e.g., *Enterobacter cloacae*, *Bacillus subtilis*), generate odorous compounds (e.g., actinomycetes, *Clostridium* spp), produce slime (e.g., *Bacillus* spp, *Klebsiella* spp) and impact human health when they encounter food (e.g., *Proteus* spp, *Salmonella* spp, molds) (Raaska et al. 2002).

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Many studies have reported spore-bearing Gram-positive bacteria *Bacillus* as the maximum protruding families for paper and paperboard contaminant. Other commonly found bacteria are Klebsiella spp, Citrobacter spp, Proteus spp, Pseudomonas spp, Salmonella spp, Enterobacter spp, Staphylococcus aureus, etc. (Vaisanen et al. 1991).

The consumption of such contaminated food through various food packaging could result in outbreak of food borne illness. Health organizations of several countries have recognized microbial content value of the paper and paperboard in food packaging but still there is no thoughtful global consideration to the bio-hazardous exposures that may arise from microbial pollution in food packaging. The regular monitoring of total bacterial count and the presence of fecal coliforms in paperboards is needed to reduce such illness. Therefore, this study aimed to determine the microbial load with their antibiotic susceptibility pattern. The outcome of this study would be helpful to reduce microbial load by suggesting good hygiene practices to all food handlers including consumers.

METHODS

Sample collection

A total of 34 food packaging paper samples from different places of Kathmandu and its vicinity were collected in steam sterilized polythene bags and transported to laboratory of Goldengate International College. Sample collection was done during study period of April to June 2019.

Microbial load detection of paper samples

Sample preparation was done by defibering method in which Ringer's solution can easily dissolve fibers containing microorganisms (Mohammadzadeh-Vazifeh et al. 2015). The bacterial load was determined by using Plate Count Agar (PCA) and fungal load was determined by using Potato Dextrose Agar (PDA) with 10-fold dilution in normal saline. One gram of each paper sample was weighed followed by serial dilution up to 10^{-5} and then inoculated aseptically on Plate Count Agar by using pour plate technique.

For selective isolation, a loopful of diluted sample (10^{-1}) was inoculated on selective media like MacConkey Agar, Mannitol Salt Agar, Eosin Methylene Blue Agar, Salmonella-shigella agar and Tryptose Citrate Bile Salt Sucrose Agar and incubated at 37°C for up to 48 hours. The isolated colonies from these media were identified by observing colony morphology followed by Gram staining and biochemical tests.

PDA plates incorporated with chloramphenicol ($0.05gl^{-1}$) were observed for fungal growth. Yeasts and molds were differentiated by observing colony morphology and microscopic study. Molds were further identified following standard microbiological procedures (*Fungal Descriptions and Antifungal Susceptibility*, n.d.).

Antibiotic susceptibility testing

Modified Kirby-Bauer disk diffusion test based on the guidelines of Clinical and Laboratory Standard Institute (CLSI 2012) method was used to evaluate the antimicrobial susceptibility pattern of the isolates to a set of antibiotics and determination of methicillin resistance *S. aureus* and ESBL producing strains. The antimicrobial agents tested for Gram negative bacteria were Ampicillin (AMP,10µg), Imipenem (IMI,10µg), Gentamycin (GEN,10µg), Cefotaxime (CTX,30µg) Ceftazidime (CAZ,30µg)), ciprofloxacin (CIP ,5µg) , Cefixime (CFM,5 µg) and Piperacillin/ Tazobactam (PIT) and for Gram positive bacteria were: Amikacin (AK,30µg), Chloramphenicol (C,30 µg), Cloxacillin (COX,10µg), Cotrimoxazole (COT,25µg) Ciprofloxacin (CIP,5µg), Erythromycin (E,15µg), Tetracycline (TE,30µg), Gentamycin (GEN,10µg).

The multidrug resistance was tested among the isolates and interpreted by using the standard guideline (Magiorakos et al. 2011)

Screening of ESBL producing and MDR organisms

ESBL producers were detected from Ceftazidime and/or Cefotoxime resistant isolates using standard combined disc-diffusion method. ESBL producer was detected by more than 5 mm distance difference in zone size between ceftazidime/ceftazidime with clavulinic acid (CAZ/CAC) and cefotaxime/ceotaxime with clavulinic acid (CTX/CEC) (CLSI 2014).

The multidrug resistance was tested among the isolated and interpreted by using the standard guideline (Magiorakos et al. 2011).

RESULTS

Among 34 paper samples collected from closed shop and open street vendors, closed shop used paper and paperboards (PPBs) whereas open street vendors extensively used reused newspaper, academic papers, office documents, printed papers and even hospital papers for food packaging. Due to this although all the samples had equal probability of getting contaminated, samples obtained from open street vendors had significantly higher microbial yield.

Microbial load detection

The food packaging paper was found to be most contaminated with an average bacterial and fungal load of 1.53×10^5 CFU/g. The obtained average microbial count obtained in open (n=21) and closed (n=13) paper samples were 3.62×10^5 CFU/g and 4.67×10^5 CFU/g respectively (Figure 1).

Microbial diversity

All the samples tested were found to be contaminated. Among the 103 microbial species identified, predominant isolates were bacteria followed by molds and yeasts (Figure 2).

Distribution of bacteria

A total of 78 bacterial isolates of 9 different species were identified, of which 4 were coliform group of bacteria, 3 were Gram negative bacteria other than coliforms and 2 Gram positive isolates. *Bacillus* spp 34 (43.59%) was the predominant isolate followed by K*lebsiella* spp 16 (20.51%). Majority of the isolates 46 (58.97%) were detected from the samples of street vendors (open retailer). Only 32 (41.03%) isolates were detected from the samples of closed retailers (Table 1).

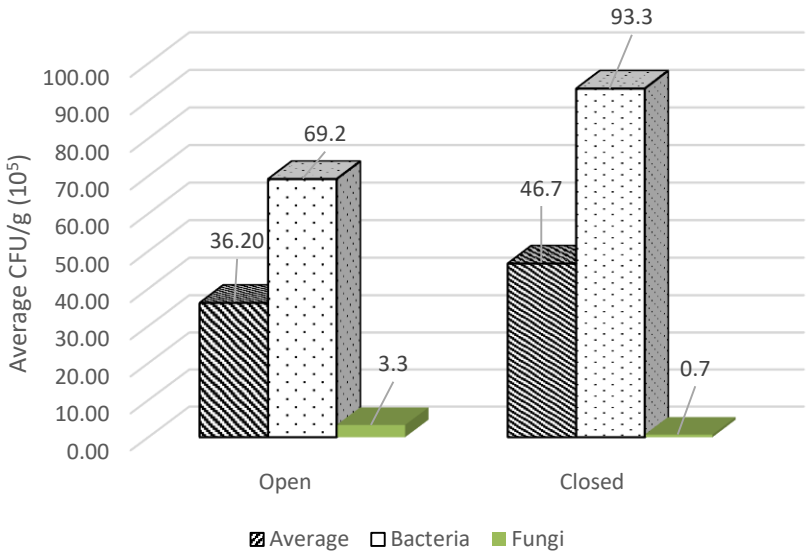

Figure 1: Enumeration of microorganism in paper samples

Distribution of fungi

Among 25 isolates of fungi isolated, 15 (60%) were molds and 10 (40%) were yeasts. Among molds identified, *Cladosporium* spp 7/15 (46.67%) was the dominant one followed by *Aspergillus* spp*, Mucor* spp and *Fusarium* spp.

Antibiotic susceptibility pattern of coliforms

The coliform isolates were most resistant against ceftazidime and ampicillin.

Antibiotic susceptibility pattern of Gram negative bacteria other than coliforms

The non-coliform isolates were resistant against ceftazidime, ampicillin and cefotaxime.

Antimicrobial susceptibility of *Staphylococcus aureus* isolates

The single isolate of *Staphylococcus aureus* was sensitive towards Gentamicin, Clindamycin, Chloramphenicol, Tetracycline and Erythromycin i.e., 1 (100%) and resistant against Cefoxitin, Penicillin and Ciprofloxacin i.e. 0 (0%).

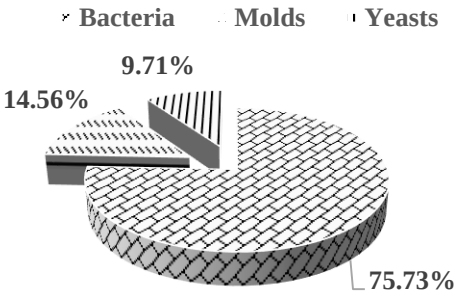

Figure 2: Microbial diversity of food packaging paper

Table 1: Distribution of bacterial isolates according to the retailer type

Category	Organisms	Retailer Type		Total (n)(%)
		Open (n)	Closed (n)	
Coliforms	*E. coli*	0	1	1(1.28)
	Klebsiella spp	11	5	16(20.51)
	Citrobacter spp	4	4	8(10.26)
	Enterobacter spp	1	5	6(7.69)
Sub-total		**16**	**15**	**31**
Gram negative bacteria other than coliforms	*Pseudomonas* spp	5	3	8(10.26)
	Salmonella spp	1	1	2(2.56)
	Proteus spp	2	0	2(2.56)
Sub-total		**8**	**4**	**12**
Gram positive bacteria	*Staphylococcus aureus*	1	0	1(1.2)
	Bacillus spp	21	13	34(43.59)
Sub-total		**22**	**13**	**35**
Total		**46**	**32**	**78**

Table 2: Distribution of fungi in paper samples

S.N.	Sample type	Sample	Fungi	Number	Percentage
1	**Open**	21	*Aspergillus* spp	2	9.52
			Cladosporium spp	3	14.28
			Mucor spp	2	9.52
			Yeasts	7	33.33
	Sub-total			**14**	
2	**Closed**	13	*Cephalosporium* spp	1	7.69
			Penicillium spp	1	7.69
			Cladosporium spp	4	30.77
			Fusarium spp	2	15.38
			Yeasts	3	27.27
	Sub-total			**11**	
	Total			**25**	

Distribution of ESBL-producing organisms

Out of total 78 isolates, 44 isolates were subjected for ESBL screening test. A total of 35(79.55%) isolates were screened positive. ESBL production by ceftazidime 5(14.28%), cefotaxime 11(31.43%) and both 19(54.28%) of them. 0(0%) were confirmed to be ESBL producer.

Table 3: Antibiotic Susceptibility Test of coliforms

Antibiotics	*Klebsiella* spp(N=16) n (%)	*Citrobacter* spp(N=8) n (%)	*Enterobacter* spp(N=6) n (%)	*E. coli*(N=1) n (%)
GEN	14 (87.5)	8 (100)	6 (100)	1 (100)
AK	16 (100)	8 (100)	5 (83.3)	1 (100)
PIT	15 (93.7)	7(87.5)	6 (100)	1 (100)
IPM	16 (100)	7 (87.5)	6 (100)	0 (0)
CTX	9 (56.2)	3 (37.5)	3 (50)	1 (100)
CFM	10 (62.5)	7 (87.5)	2 (33.3)	1 (100)
CIP	16 (100)	8 (100)	6 (100)	1 (100)
CAZ	14 (87.5)	0 (0)	0 (0)	1 (100)
AMP	1 (6.25)	0 (0)	0 (0)	1 (100)

GEN-Gentamicin, AK-Amikacin, PIT-Piperacillin/Tazobactam, IPM-Imipenem, CTX-Cefotaxime, CFM-Cefoxime, CIP-Ciprofloxacin, CAZ-Ceftazidime AMP- Ampicillin

Table 4: Antibiotic Susceptibility Test of Gram-negative bacteria other than coliform

Antibiotics	*Pseudomonas* spp(N=8) (n%)	*Salmonella* spp(N=2) (n%)	*Proteus* spp(N=2) (n%)
GEN	7 (87.5)	2 (100)	2 (100)
AK	8 (100)	2 (100)	2 (100)
PIT	7 (87.5)	2 (100)	1 (50)
IPM	7 (87.5)	2 (100)	2 (100)
CTX	2 (25)	1 (50)	0 (0)
CFM	7 (87.5)	2 (100)	0 (0)
CIP	8 (100)	1 (50)	2 (100)
CAZ	2 (25)	1 (50)	1 (50)
AMP	1 (12.5)	1 (50)	0 (0)

GEN-Gentamycin, AK-Amicakin, PIT-Pipercillin/Tazobactam, IPM-Imipenem, CTX- Cefotaxime, CFM- Cefoxime, CIP-Ciprofloxacin, CAZ-Ceftazidime, AMP- Ampicillin.

Table 5: Distribution of ESBL-producing organisms

Isolate	Screened positive			Confirmed
	CAZ only	CTX only	Both	
Klebsiella spp (n=16)	0	9	5	2
Citrobacter spp (n=6)	1	1	3	0
Salmonella spp (n=2)	0	0	1	0
Enterobacter spp (n=6)	4	0	2	1
Pseudomonas spp (n=8)	0	1	7	1
Proteus spp (n=2)	0	0	1	0

Table 6: MDR profile of the isolates

Resistance towards drug	Number of isolates	Number of Antibiotic classes	Organism
AMP, CTX, PIT	1	3	*Klebsiella* spp
AMP, CAZ, CFM, PIT, CTX	1	3	*Enterobacter* spp
AMP, GEN, CAZ, CFM, CTX	1	3	*Klebsiella* spp
AMP, CFM, IPM	1	3	*Pseudomonas* spp

Multidrug resistance

Two species of *Klebsiella* spp, one *Enterobacter* spp and one *Pseudomonas* spp were confirmed to be multi drug resistant (Table 6).

DISCUSSION

Paper being biodegradable and environment friendly, they are the most commonly used food packaging materials in comparison to plastic and other method of food packaging. Paper packaging is not only prevalent among street vendors even sweet shops, bakeries, etc. also use them commonly. As food remains in contact with these papers, microbiological study of them can be considered as an important aspect as it's a matter of health of general people.

During this study, the total number of 34 food packaging paper samples were collected from different places of Kathmandu valley during 3 months of study from April to June 2019. Each of the 34 samples yielded microbial growth. This may have occurred as a result of contaminated raw materials used in paper production, during processing of raw materials, during transportation and during handling. The microbes were enumerated, isolated and identified for microbial analysis.

The average bacterial load obtained from defibering method was (2.65×10^2 -5.4×10^6) CFU/g which was comparable with study performed by Mohammadzadeh-Vazifeh et al. (2015) which was in the range of (0.2×10^3 to $> 1.0\times10^5$) CFU/g and comparatively less than studied

by Rana et al. (2019) which was in the range of (1.9×10^{8}-7.5×10^{8}) CFU/g.

Higher number of bacterial isolates were detected with range between (2.7×105-3.01×105) CFU/g which exceed the given permittable range of 2.5×102 CFU/g for paper materials used for food packaging defined by FDA (Food and Drug Administration) (Sood and Sharma 2019).

Lower number of isolates were found to be at the range of 0.2×102 to 0.4 ×102 CFU/g which is accordance to the value defined by FDA and can be considered as safe for packing food.

The total number of microbial isolates detected were 103 of which *Bacillus* spp (43.59%) was the predominant bacteria followed by *Klebsiella* spp. This may be due to their ubiqutous and spore forming nature. Study conducted by Sood and Sharma (2019) also reported *Bacillus* spp as dominant bacteria. In paper industry these *Bacillus* spp are primary organisms to accumulate slime by themselves which starts by formation of monomolecular layer. These bacteria also enhance growth of secondary organisms such as *Klebsiella* spp and *Pseudomonas* spp (Blanco et al. 1996). The growth of other bacteria isolated also have potential to cause food borne illness leading to complications (Bennett et al. 2013).

Molds like *Cladosprium*, *Aspergillus*, *Fusarium* were identified which have potential to produce mycotoxin directly affecting the consumers' health (*Mycotoxins: Risks in Plant, Animal, and Human Systems*, 2003).

Ciprofloxacin (42/43) and Amikacin (42/43) were most effective and ampicillin (39/43) was most resistant antibiotics towards Gram negative bacteria. No MRSA isolates and four ESBL producers *Klebsiella* spp (2), *Pseudomonas* spp (1) and *Enterobacter* spp (1) were confirmed from paper samples. Similarly, all the ESBL producers were MDR. Presence of MDR isolates suggests spread of community-associated (CA) MDR bacteria related to high mortality and morbidity (van Duin and Paterson, 2016). The high resistance to the commonly used antibiotics may be due to random source of the papers including hospital. This result indicates that potential pathogens are found in food packaging papers which can be threat to health of consumers.

CONCLUSION

All of the 34 samples were contaminated with bacteria and fungi among which *Bacillus* spp was the most predominant bacteria. Also, the bacterial load in open paper used by street vendors exceeded the permissible limit provided by FDA.

Mostly reused newspaper, academic papers, office documents, printed papers and even hospital report papers were used as packaging materials. Microbial contamination depends on the type of papers used by them. The presence of such microbial contaminants is uncommon and unsafe for human health. So, the reliable safe supply of food is important for people's general health. The result confirmed that the microbial contamination of paper-based foodstuff may impose health hazard or infection.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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