Journal of Cell and Tissue Research Vol. 14(2) 4405-4408 (2014)
(Available online at www.tcrjournals.com)
ISSN: 0973-0028; E-ISSN: 0974-0910

Original Article

IDENTIFICATION OF BIOFILM AND EXTENDED SPECTRUM B-LACTAMASES (ESBL) PRODUCING ISOLATE OF *KLEBSIELLA PNEUMONIA* FROM TAMILNADU

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Received: April 24, 2014; Accepted: June 20, 2014

Abstract: ***Prevention of food-borne illness is an important public health concern worldwide. Consumption of raw or undercooked unhigeneic meat and eggs linked to health risk to individuals with compromised immune system. In the recent years, multi drug resistant Escherichia coli and Klebsiella pneumoniae strains pose a great threat to many countries. The aim of the present study was to evaluate the presence of drug resistant K. pneumonia from local meat chains. Chicken, beef, goat tissue samples from small scale abattoir and commercially available processed meat sausages were tested for the presence of multi drug resistant K. pneumonia. Among the tested meat samples, highest occurrence of β-lactamase producing K. pneumonia was observed in goat meat sample (70%), bovine (60%) and chicken (10%). The prevalence of extended-spectrum β-lactamase genes identified by the amplification of SHV, TEM, CTXm and OXA specific primers by PCR. TEM type was observed in all meat isolates having strong or medium bio film production, followed by CTXm. Antibiotic resistance was found to be more in goat isolates with 68.5%, followed bovine isolates with 56.6% and chicken isolates with 46%. In our study, the strong biofilm producing individual isolates (41%) had at least three of the ESBL genes with higher percentage of antibiotic resistance (>70%) than weak or moderate biofilm producing isolates.***

Key words: ß-lactamase, *Klebsiella pneumonia*

INTRODUCTION

Klebsiella pneumoniae is an emerging pathogen. In humans it has been associated with various ailments such as urinary tract infection, septicemia, respiratory tract infection and diarrhoea. In animals, it causes clinical infections in dog, horse, cattle, cats, and pigs [1]. In the recent years, many studies confirmed the increased frequency of multidrug resistant *Klebsiella pneumoniae* and *E. coli* isolates with extended spectrum â-lactamase (ESBL) from food source particularly in meat samples worldwide [2,3]. ESBL producing *Enterobacteriaceae* prevalence rate varies between country to country depending upon the level of hygienic measures they follow. "ESBL phenotype" has been mainly described in TEM-, SHV-, CTX-M, GES, and VEB families into class A β-lactamases, and OXA-ESBLs into class D â-lactamases. The TEM- and SHV-ESBLs were predominant in the ESBL landscape over the 1980s and 1990s in the past century, mainly associated with outbreaks in hospitals involving *Klebsiella pneumoniae* and to a lesser extent in *Escherichia coli* and other Enterobacteriaceae, whereas the

CTX-M were less prevalent. Delay in suitable antimicrobial treatment of ESBL producing *K. pneumonia* infections were associated with increased morbidity and mortality and prolonged infection management [4]. Biofilm formation in *E. coli* and *K. pneumonia* allows the strains to persist a long time, it is likely that they develops mechanisms to multiple drug resistance and immune evasion [5]. Little is known about the antimicrobial resistance mechanisms in *K. pneumoniae* from clinical isolates in India however the prevalence of this multi drug resistant bacterium was not evaluated from meat products. Hence the present study was aimed to determine the occurrence of multi drug resistant *Klebsiella pneumoniae*, their ESBL types and biofilm production ability in meat samples.

MATERIALS AND METHODS

A total of thirty random raw meat samples (Goat n=10, beef n=10 and chicken n=10) were obtained from 11 local abattoirs cum retail outlets in different locations of Salem and Namakkal District, Tamilnadu, between January and April, 2012. Also we collected 20 processed meat samples from various super markets (Goat n=7, beef n=7 and chicken n=6) during this time period. The tissue samples were transported to the laboratory on ice and processed for the bacterial isolation as per Brown et al. [6].

Antimicrobial susceptibility test: The antimicrobial susceptibility to other antibiotics commonly used in clinical treatments was evaluated by the standard disk diffusion method standard agar dilution on Mueller-Hinton agar. Antibiotic susceptibilities were determined for the following antimicrobial agents: Tobramycin (TB), Norfloxacin (NX), Cephotaxime/ Cefotaxime (CTE/CE), Methicillin (M), Kanamycin (K), Co-trimaxazole (Co), Nalidixic acid (NA), Ciprofloxacin (Cip), Tetracycline (T) and Vancomycin (VA).

Biofilm production assay: Biofilm production was examined as per Freeman et al. [7], by using Congo Red Agar (CRA) medium. CRA medium was prepared with brain heart infusion broth (HiMedia, India) (37 g/L), sucrose (50 g/L), Agar (10 g/L) and Congo red indicator (8 g/L). CRA plates were inoculated with individual bacterial isolates and incubated at 37ºC for 24 hrs aerobically. Black colonies with a dry crystalline consistency indicated biofilm production. Strong biofilm formation was indicated by black colonies with a dry crystalline consistency. A darkening of the colonies with the absence of dry crystalline colony morphology was considered as moderate result. Weak slime producers usually remained pink, however occasional darkening at the centres were observed in few isolates.

Assay for beta lactamase production: Beta lactamase production was assayed using the method of Lateef et al. [8]. Broth culture of the test organism was spot inoculated on to Mueller-Hinton agar and 1% starch and then incubated overnight at 37^0C. The plates were then flooded with sterile phosphate buffered saline containing potassium iodide, iodine and penicillin. Beta lactamase production was assessed by the presence of clear colourless zones around the bacterial growth. All the bacterial isolates were tested for the production of beta lactamases.

Characterization of β-lactamase genes: With the aim of characterizing the β-lactamase (bla) genes involved in the ESBL production, PCR was done with TEM, OXA, CTX-m and SHV specific primers described earlier Hong Fang [9]. (2008). Individual bacterial isolates were grown overnight on nutrient agar plates (Himedia, Mumbai) at 37°C. Individual colonies were suspended in 250 µl of sterile nuclease free water and boiled for 10 min and 2.5µl aliquot was used as a template DNA for PCR. The PCR amplification was carried out in a final volume of 25 µl, containing 2.5 µl of buffer (10X Taq buffer), 2.5µl 25mM MgCl2, 1 µM of SHV, CTXm, OXA and TEM sense and sntisense primers (Sigma-Aldrich), 0.2 mM of dNTP mix and 1U of Taq-polymerase (Thermo Fisher Scientific Inc). The amplification conditions used were as described earlier. The PCR amplicons were analyzed by electrophoresis on a 1.5% Agarose gel, containing ethidium bromide (0.2 mg/ml), in the presence of an appropriate DNA molecular weight marker.

RESULTS AND DISCUSSION

Identification and sensitivity profiles of *Klebsiella penumoniae* isolates: In this study, we isolated *K. pneumoniae* with different patterns of antibiotic resistance, ESBL genes with biofilm producing capacity. In particular, *K. pneumoniae* isolates from ruminants such as goat and bovine meat samples revealed the higher levels of antibiotics

Table 1: Antibiotic resistance among *Klebsiella pneumonia* isolates from meat samples

S.No	Isolates name	Antibiotics										% of Resistance
		TB	NX	CTE	T	K	Co	NA	CIP	M	VA	
1.	B1	I	R	R	R	R	I	I	R	R	R	56.6
2.	B2	I	R	I	R	I	S	I	R	R	R	
3.	B3	S	R	R	R	I	I	R	I	S	I	
4.	B6	I	R	R	R	R	I	R	R	R	R	
5.	B8	S	R	R	R	I	I	R	I	R	R	
6.	B10	S	I	I	R	S	R	I	I	R	R	
7.	C2	S	I	I	R	S	R	I	I	R	R	46
8.	C5	S	S	I	R	S	I	I	S	S	R	
9.	C8	I	I	S	R	I	R	R	I	R	R	
10.	C9	S	I	R	R	I	I	R	R	S	R	
11.	C10	S	R	I	R	R	I	R	R	R	R	
12.	G2	S	R	I	R	S	I	R	R	R	R	68.5
13.	G3	S	R	I	R	I	R	R	R	R	R	
14.	G4	S	R	I	R	I	R	R	I	R	R	
15.	G6	S	R	R	R	R	R	R	I	R	R	
16.	G7	S	R	I	R	I	I	R	R	R	R	
17.	G8	S	R	R	R	I	I	R	I	R	R	
18.	G10	S	I	I	R	I	I	S	I	R	R	
% of Resistance		0	66.6	38.8	100	22.2	33.3	66.6	44.4	83.3	94.4	

Table 2: Correlation of biofilm production of *Klebsiella pneumonia* isolates, sensitivity test and with the presence of TEM, SHV, OXA and CTX-M type ESBL genes

S.No	Isolates name	% of antibiotic resistance	Result for biofilm	ESBL antibiotic resistance			
				TEM	SHV	OXA	CTXM
1.	B1	70	S	+	+	-	+
2.	B2	50	-	-	-	-	-
3.	B3	40	-	-	-	-	-
4.	B6	80	S	+	+	+	+
5.	B8	60	M	+	-	-	-
6.	B10	40	-	-	-	-	-
7.	C2	40	-	-	-	-	-
8.	C5	20	-	-	-	-	-
9.	C8	50	W	+	-	-	-
10.	C9	50	W	-	-	-	-
11.	C10	70	S	+	+	+	+
12.	G2	60	M	-	+	+	-
13.	G3	70	S	+	+	+	+
14.	G4	60	M	+	-	-	+
15.	G6	80	S	+	-	+	+
16.	G7	60	W	-	-	-	+
17.	G8	60	M	-	-	-	-
18.	G10	30	-	-	-	-	-

resistance (68.5% and 56.6% respectively), whereas chicken samples found to harbour isolates with 46% resistance. Recent report from Spain and Nigeria suggests that the meat samples were contaminated with different kinds of bacterial species [3,10] also patterns of resistance among varies among bacterial isolates from animal origin in various countries [11].

In our study, apart from *Klebsiella pneumoniae* (36%), we also observed *Escherichia coli* (22%), *Campylobacter jejuni* (8%), *Salmonella typhi* (8%) and *Staphylococcus aureus* (6%) in the 50 meat samples analysed. Since the *K. pneumoniae* was isolated in large proportion, further study was focused on only with *Klebsiella* isolates.

In our study we observed higher percentage of resistance to tetracycline (100%), vancomycin (94.4%), methicillin (83.3%), norfloxacin (66.6%) and nalidixic acid (66.6%) antibiotics was observed. However higher sensitivity was observed to tobramycin (77.77%), differential susceptibility also observed to kanamicin, cephotaxime/cefotaxime and co-trimaxazole. The susceptibility test of meat isolates has shown the resistance to at least one antimicrobial agent was observed in all 18 klebsiella isolates (Table 1).

Characterization of ESBL genes and biofilm formation: Presence of one or more β-lactamase genes were identified in 10 (55 %) of the 18 meat isolated strains. High level prevalence of the TEM type was found in 8 isolates (44.4%) followed by a CTXm type 7 isolates (38.8), SHV and OXA was found in 5 isolates (27.7%). TEM was the most frequent ESBL type found alone or in combination with CTXM â-lactamase (55.5%). CTX-M was always found together with a TEM type beta-lactamase gene. The occurrence of SHV and OXA type of genes found to be less proportion than the TEM or CTXM genes. The existence of SHV and OXA type of genes were found with the combination either TEM or CTXM genes. It is also observed that, 8 (44.4%) *K. pneumoniae* isolates does not have any of these ESBL genes (Table 2). In our study, we have found that the majority of biofilm producing bacteria was from goat isolates (85.71%), beef and chicken isolates had comparatively less (50% and 60% respectively) with higher antibiotic resistance. Interestingly our results agree with the previous report [12], we have observed higher antibiotic resistance in biofilm producing bacteria (70 %-80 %) than non-biofilm producers (20%-50%). Whereas medium and weak biofilm producing bacterial isolates contains 50-60% resistance. In general, bacterial isolates produc ing biofilm are basically more resistant to antimicrobial agents than non-biofilm producing cells, high antibacterial concentrations upto 1,000 fold are required to control biofilm producers [13]. In addition to the ability to increased resistance to antimicrobial agents, biofilm forming bacteria is an efficiently avoids the complement pathways and phagocytosis [5]. Remarkably in this study, bacterial isolates with strong biofilm has shown higher resistance to several antibiotics than medium or weak biofilm forming isolates.

In conclusion, biofilm physiology of Klebsiella isolates from meat samples correlates well antibiotic resistance and ESBL production. Although the amount of samples and location analysed in this study were limited, however it represents the prevalence of multidrug resistant bacteria from poor hygiene meat products. Nationwide multi drug resistance surveillance may give a clear picture to compare the quality of food products from different origin.

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