International Journal of Current Science Research
www.drbgrpublications.in

Volume 7; Issue 1; January-2021; pp 2070-2077. ISSN: 2454-5422

Klebsiella pneumoniae in meat and application of specific phage

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Abstract

Food contamination is a serious issue because it results in foodborne diseases. It can be microbial or environmental. Meat is a good medium for the multiplication of bacteria that cause food-borne diseases. *K. pneumoniae* is a colonizing devious pathogen of humans and animals, and a widespread contaminant of retail meat. Control of those food-borne pathogens has been done using various natural or chemical food preservatives. Chemical preservatives are not preferred due to the side effects they cause. Bacteriophages are viruses that used as biocontrol and bio preservation agents. They are really effective and specific against their bacterial host without a side effect on the intestinal microflora.

Keywords: Bacteriophages, *K. pneumoniae*

Introduction

Meat is an excellent protein source in human diet and also highly susceptible to microbial contaminations; it causes human spoilage and foodborne diseases, contributing to economic and health losses (Komba *et al.*, 2012). While microorganisms do not include the muscles of healthy animals, meat tissue is infected during the various stages of production, ranging from slaughter to transport (Ercolini *et al*., 2006).

K. pneumoniae sp. Pneumoniae is a Gram-negative bacterium that causes a number of diseases, including urinary tract and soft tissue infections, bacteremia, and pneumonia. In developing countries. *K. Pneumoniae* has historically been seen as an

opportunistic pathogen responsible for nosocomial infections (Podschun and Ullmann, 1998).

K. pneumoniae is a colonizing pathogen of humans and animals, and a widespread contaminant of retail meat (Kim *et al.*, 2007b). Bacteriophages are a kingdom of viruses that destroy bacteria. It's a combination of the word “bacteria” and a Greek word “phagein” meaning to eat. They cannot attack cells of more compound organisms because of differences in cell-surface components. They are not lethal or infectious to humans or other mammals, phages are safe, a normal aspect of the human environment and are commonly consumed via foods (farmed freshwater fish, chicken, oil sardines, raw skim milk, cheese, yogurt, etc) and drinking water (Rehna *et al*., 2016). They used as a preservatives in fragile manufactured foods. As they are recorded to lyse their hosts at temperatures as low as 1 C, phages are excellent food biopreservation agents. Also refrigerated foods (especially psychrotrophic bacteria) restrict their growth (FDA, 2002).

Aim and Objects

- Isolation and enumeration of *K. pneumoniae* in meat samples.
- Isolation specific phage from sewage samples.
- Application of phage to control *K. pneumoniae* in meat.

Materials and Methods

Processing of samples

Ten grams of each meat sample in 90 mL buffered peptone water were mixed and homogenized in sterile electric blender. Make a filtration and take the filtrate. One mL of this filtrate was subjected to ten-fold dilution series of homogenates samples were prepared and inoculated onto nutrient agar plate medium (FDA, 2002).

Isolation and enumeration of *K. pneumoniae*

Isolation was carried out by spreading 0.01 mL from each sufficient dilution of each meat samples onto plates of Xylose Lysine Deoxycholate Agar (XLD) and Eosin Methylene blue (EMB) incubated at 37°C for 24 h. typical colonies which appear large yellow colonies on XLD agar and pink purple color on EMB agar were counted then picked (FDA, 2002).

Cultural characteristic and gram's staining for bacterial isolate

Morphological characteristics of colonies (size, shape, elevation form, pigmentation and opacity) developed after incubation on selective agar plates were carefully studied and recorded. Gram's staining was performed as per procedures described by Merchant and Packer, (1969) to determine the size, shape and arrangement of bacteria.

Identification of bacterial isolates

It was done using VITEK II automated system (Funke *et al*., 1998).

Isolation of bacteriophages

Biologically stock phage lysates were obtained according to method described by Adam's, (1959) using the single plaque isolation method based on the morphological characters like diameter, halo, turbidity and shape**.**

Propagation of the single phage lysates

A large amount of high titer phage stock was obtained by Liquid culture propagative method (Adam's, 1959)

Purification and concentration of *K. pneumoniae* phage

The propagated *K. pneumoniae* phage isolate was purified and concentrated using the alternative differential centrifugation method (Figrski and Christensen, 1974). After twice cycles, the pellets were re-suspended in 300 µL CM buffer (1M Tris buffer; 2.5g/L MgSO4.7H2O; 0.735g/L CaCl2; 0.05g/L gelatin; pH 7.5).

Examination of phage morphology by TEM

The morphology of *K. pneumoniae* phage lysate was examined using Transmission Electron Microscopy.

Application of bacteriophage to control pathogenic bacteria in meat at room temperature

Non-sterilized grinded fresh meat was taken from the butcher and placed in sterile bags then put in a separated container with ice for storage. Samples were transported directly to the laboratory. Three treatments were grouped applied as follow:

1. Ten g of non-sterilized fresh meat was left without any treatment after estimation *K. pneumoniae* bacteria on it.
2. Ten g of non-sterilized fresh meat was treated with 1 mL of *K. pneumoniae* phage with MOI=100).
3. Ten g of non-sterilized fresh meat was treated with 1 mL of *K. pneumoniae* phage with MOI=100) followed by addition 1 g spices.

- Spices were mixed with equal quantities of cumin, chili, pepper ground and coriander then 1 g was taken.

Results

Table 1. Log number of *K. pneumoniae* on different meat products.

Fresh meat & meat products	Specimen No	***K. pneumoniae*** On XLD agar
Burgers	1	2.40 ± 0.00^{fg}
	2	2.33 ± 0.145^{fg}
	3	2.50 ± 0.10^{f}
	4	1.80 ± 0.057^{i}
Fresh meat	1	3.60 ± 0.057^{c}
	2	3.20 ± 0.057^{d}
	3	1.67 ± 0.033^{i}
	4	3.17 ± 0.033^{d}
Luncheon	1	4.63 ± 0.033^{a}
	2	4.03 ± 0.033^{b}
	3	3.17 ± 0.088^{d}
	4	2.23 ± 0.033^{g}

Table 2. Morphological characteristics of *K. pneumoniae* isolate.

Test	***K. pneumoniae*** on XLD agar
Family	*Enterobactericeae*
Colony Shape	circular
Colony Size	2-3mm
Colony Elevation	convex
Colony Color	yellow
Gram reaction	Gram negative
bacterial Shape	rods

Table 3. Biochemical details for *Klebsiella pneumoniae ssp pneumoniae* obtained by VITEK II automated system.

Bio number	*6605734373564010*
organism	*Klebsiella pneumoniae ssp pneumoniae*
probability	*99%*
confidence	*Excellent identification*

2	*APPA*	-	*3*	*ADO*	+	*4*	*PyrA*	+	*5*	*IARL*	-	*7*	*dCEL*	+	*9*	*BGAL*	+
10	*H25*	-	*11*	*BNAG*	-	*12*	*AGLTp*	-	*13*	*dGLU*	+	*14*	*GCT*	-	*15*	*OFF*	+
17	*BGLU*	+	*18*	*dMAL*	+	*19*	*dMAN*	+	*20*	*dMNE*	+	*21*	*BXYL*	+	*22*	*BAIap*	-
23	*ProA*	-	*26*	*LIP*	-	*27*	*PLE*	+	*29*	*TyrA*	+	*31*	*URE*	+	*32*	*dSOR*	-
33	*SAC*	+	*34*	*dTAG*	+	*35*	*dTRE*	+	*36*	*CIT*	+	*37*	*MNT*	+	*39*	*5KG*	-
40	*ILATK*	+	*41*	*AGLU*	-	*42*	*SUCT*	+	*43*	*NAGA*	-	*44*	*AGAL*	+	*45*	*PHOS*	+
46	*GIyA*	-	*47*	*ODC*	-	*48*	*LDC*	+	*53*	*IHISa*	-	*56*	*CMT*	-	*57*	*BGUR*	-
58	*O129R*	+	*59*	*GCAA*	-	*61*	*IMLTa*	-	*62*	*ELLM*	-	*64*	*ILATa*	-			

Table 4. Plaque morphology of purified isolated phage.

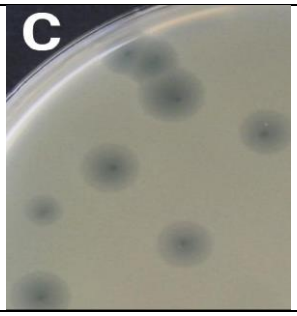
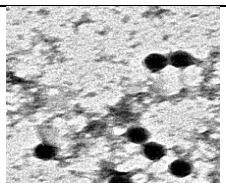
Isolated phage	**Plaque Size (mm)**	**Plaque morphology**	photo plate **of *K. pneumoniae* plaques**
K. pneumoniae	6.0	Circular, clear with center, With large halo	

Table 5. Morphological properties and prospected family of phage isolate by TEM.

Phage	**Head (nm)**		**Tail (nm)**		**Prospected family**	***K. pneumoniae* Micrograph by TEM**
	size	**shape**	**Size**	**shape**		
K. pneumoniae	69.2	Icosahedral	---	----	*Podoviridae*	

Application of *K. pneumoniae* phage in meat

Bacterial numbers in non-phage treated fresh meat were increased by long preservation time it was found that *K. pneumoniae* bacterial number at zero time was 4.13 log CFU/g after 12 h it was 5.92 log CFU/g, after 24 h it was 6.17 log CFU/g, after 48 h it was 7.35 log CFU/g and 8.90 log CFU/g after 72 h. After adding phage to minimize bacterial contamination, it was observed that the count of *K. pneumonia* bacteria were reduced after 12 h from the addition by 1.47 log CFU/g, after 24 h by 1.8 log CFU/g, after 48 h by 1.5 log CFU/g and after 72 h by 3.28 log CFU/g. After addition of phage with spices mixture, *K. pneumoniae* cells were reduced after 12 h from the addition by 1.51 log CFU/g, after 24 h by 1.90 log CFU/g, after 48 h 1.63 log CFU/g and by 3.34 log CFU/g after 72 h. It means that Spices were increased the reduction in bacterial counts comparing to not addition. As showed in (Table 6).

Table 6. Increasing and reduction in Log numbers of *K. pneumoniae* after and before application of phage at room temperature.

Bacterial isolate	*K. pneumoniae*				
Preserved time / Treatment	Zero	12 h	24 h	48 h	72 h
T_1	4.13 ± 0.029^{e}	5.92 ± 0.06^{d}	6.1 ± 0.68^{c}	7.3 ± 0.007^{b}	8.90 ± 0.17^{a}
T_2	4.13 ± 0.029^{e}	4.45 ± 0.29^{c}	4.37 ± 0.013^{d}	5.85 ± 0.3^{a}	5.62 ± 0.24^{b}
T_3	4.13 ± 0.029^{e}	4.40 ± 0.42^{c}	4.26 ± 0.5^{d}	5.72 ± 0.36^{a}	5.56 ± 0.02^{b}

- (a, b, c,...) Average in the same row having different superscripts are differ significantly.
T_1=untreated fresh meat.
T_2=treated meat with *K. pneumoniae* phage.
T_3= treated meat with *K. pneumoniae* phage+ spices mixture.

Discussion

Phage was prepared with 10^{10} PFU/mL and applied to preserve fresh meat at room temperature. It was found that Phage was effective After 72 h from the application of phage at room temperature. The numbers of *K. pneumoniae* on non-sterilized meat decreased by 3.28 log CFU/g, when adding phage with food spices, *K. pneumoniae* numbers decreased by 3.34 log CFU/g. So that food spices contributing in reduction of bacterial contamination because it contain antimicrobial agents. Reduction was high as using high MOI (100). The data ensure that high MOI (100) showed maximum control of bacterial pathogens. On meat 10^{6} MOI was used to control E.

Coli O157:H7 also on melon 10^4 MOI was used to control Salmonella (Leverentz *et al*., 2001; O'Flynn *et al*., 2004; Bigwood *et al*., 2008) Investigated that after 24 h there were 4.5 and > 5.9 log cm2 differences in Salmonella counts between treated and untreated samples on cooked and raw meat.

Acknowledgement

The authors are thankful to Dr. Khaled El-dougdoug (Prof. of Virology, Agri Faculty, Ain Shams University) for continuous encouragement in accomplishing this work.

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