ORIGINAL RESEARCH

Does Over-colonization of *Klebsiella pneumoniae* in the Gut Cause Obesity?

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Abstract

Background and objectives: Gut microbes have been implicated in human weight gain and involve a few species of the Enterobacteriaceae family such as *Klebsiella pneumoniae*. We have tried to explore the effect of early colonization of the *K. pneumoniae* and subsequent eradication through bacteriophage therapy in rat pups on weight gain and loss.

Materials and methods: Three pairs of rats selected for mating were grouped separately. Group I having five pups were kept on a sterile diet. Five pups each belonging to group II and III were fed with *K. pneumoniae*. At the end of 10th week, the pups belonging to the group III were fed with *K. pneumoniae*-specific phages for 8 weeks. At the end of 30th week, group III were again fed with the bacterium, while group II received bacteriophage therapy for the next 8 weeks. The weight of each of the pups was noted every Monday of the week till the completion of the study.

Results: There was significantly higher weight gain (p <0.001) in the rats colonized by the bacterium (50% higher) than those without the colonization by *K. pneumoniae* by the end of the seventh week. When the bacterium was eradicated using a specific bacteriophage cocktail orally, the mean weight decreased and became almost similar to that of the control rats in about 12 weeks.

Conclusion: The bacterial species *K. pneumoniae*, which is a saprophyte with voracious metabolic activities, may lead to more harvesting of energy from the food and in turn lead to obesity.

Keywords: Bacteriophage therapy, Charles Foster rats, *Klebsiella pneumoniae*, Obesity.

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Introduction

There has been a substantial increase in the prevalence of obesity among rural residents and older Indians since 1975.[1] Consequently, obesity-related comorbidities, i.e., cardiovascular diseases, type-II diabetes, osteoarthritis, gallbladder diseases, backache, obesity-associated cancers, hypertension, breathlessness, including psychological disturbances, are on the increase.[2] The etiopathogenesis of obesity is multifactorial. Various factors like genetics, economic, psychological, physical exercise, diet, reproductive, and pharmacological etc., have been proposed to contribute to the genesis of obesity.[3–9] The human metagenome is considered a composite of genes of *Homo sapiens* and those trillions of microbes colonizing the body.[10] The introduction of antibiotics for the last 70 years may have induced obesity as it affects the gut microbiome.[11] Gut microbiota exerts many functions, such as stimulating effect on the intestinal epithelium, leading to the appearance of microvilli and mobility affecting the quantity of energy absorbed.[11–14]

It has been demonstrated that germ-free mice eat more but gain less weight than conventionally reared mice, indicating the importance of gut microbiota and weight gain.[15] The transplantation of gut microbiota from discordant human twins to the two groups of germ-free mice ensued into the expression of the donor's respective phenotypic character again shows the significance of the type of gut microbiota on weight gain.[16] Therefore, the new term has been coined as "Infectobesity."[17] Gut dysbiosis in terms of preponderance of either Firmicutes or Bacteroides or Actinobacteria has been proposed in the causation of obesity; however, phylum-level differences of gut microbiota between lean and obese individuals may not be universally real.[18] To establish the concept of "Infectobesity", we should try to determine etiological agent/s causing obesity.[19] *Chlamydiae trachomatis, Selenomonas noxia*, *Helicobacter pylori, Chlamydophila pneumoniae*, and viruses, e.g. certain adenovirus, canine distemper virus, Borna disease virus, enteroviruses, and Herpes simplex 1 and 2, etc., have been implicated in the genesis of obesity.[20,21]

In a pilot study, we cultured stool samples from five lean and obese subjects. Interestingly, all the five stool samples from obese subjects yielded *K. pneumoniae* as a predominant growth, while this bacterium was absent from the samples of leans subjects (unpublished data). A few Chinese studies claim that *K. pneumoniae* and *Enterobacter cloacae* have been associated with nonalcoholic fatty liver disease in human being[22] and enhanced subcutaneous fat accumulation in rats.[23]

With this background, we have planned to explore the effect of *K. pneumoniae* colonization and its eradication by using its specific bacteriophage cocktail on weight gain or loss in an animal model.

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Materials and Methods

The Institute Animal Ethics Committee of Banaras, Hindu University, approved the experimental protocol (Dean/2018/C.A.E.C./821 dated August 29, 2018). This study was carried out from July 2019 to May 2020.

Study Design

Three pairs of Charles Foster adult male and female rats were selected for mating. The selected rats did not have prior colonization of *K. pneumoniae* in their gut, as proved by culturing stool samples on MacConkey agar. All three pairs of rats were fed *ad libitum* with a standard chow diet and sterile drinking water. The animals were divided into three groups:

Group I: This group comprised the five pups delivered from the mother who was only on a standard chow diet and sterile drinking water.

Group II: In this group, the five pups delivered from the mother who was given *K. pneumoniae* in the drinking water at the final concentration of 10^9 CFU/mL since put for mating were included. The bacterial feeding was continued up to 10 weeks after birth. The bacteriophage cocktail was initiated at the concentration of 10^{12} PFU/mL at the end of 30th week and continued for further 2 weeks.

Group III: This group consisted of the four pups delivered from the mother who was given *K. pneumoniae* in the drinking water at a final concentration of 10^9 CFU/mL since put for mating. The bacterial feeding was continued up to 10 weeks after birth. The bacteriophage cocktail at a concentration of 10^{12} PFU/mL was initiated at the end of 10th week and continued for further 2 weeks. At the beginning of 31st week, the rats belonging to this group were again fed with *K. pneumoniae* at a final concentration of 10^9 CFU/mL for 8 weeks.

The weight of each of the experimental animal was recorded every Monday of the week. Blood samples were collected from the retro-orbital vein of the rats at the end of 30th week to estimate serum urea, creatinine, cholesterol, triglyceride, high-density lipoprotein (HDL), low-density lipoprotein (LDL), very-low-density lipoprotein (VLDL), serum glutamic oxaloacetic transaminase (SGOT), serum glutamic pyruvic transaminase (SGPT), total bilirubin (TB), and direct bilirubin. These parameters were estimated by using the Johnson & Johnson-4600 Chemistry System Autoanalyzer (Mumbai, India) working on the principle of Dry Chemistry Technology.

Isolation of *K. pneumoniae* Strains

A total of 100 isolates of *K. pneumoniae* were isolated from clinical and environmental samples. These isolates were used to test specific bacteriophage activity in order to pick up the three most lytic phages for the strain used for feeding the rats. One isolate of *K. pneumoniae* (*Kpn*ob01) from an obese individual was isolated and identified. This isolate was suspended in normal saline at a concentration of 10^9 CFU/mL and was given to the animals in the study through drinking water.

Isolation of Bacteriophages

For isolation of respective bacteriophages, water specimens in the volume of 100 mL were collected from different sources like hospital sewage, river Ganga, ponds, sewer of the municipal corporation, etc. The water was centrifuged, and the supernatant was collected and treated with 1% chloroform for 10 minutes. The lawn culture of the different isolates of *K. pneumoniae* was brought into the log phase by incubating for 4 hours in a 90 mm Petri plate. The chloroform-treated water was poured on each plate in the volume of 2 mL and incubated overnight at 37°C for plaque formation. If plaques were not seen, the surface of the plate was washed with 5 mL Tris-Magnesium chloride buffer (pH 7.0). The washing obtained was centrifuged and treated with 1% chloroform to lyse the bacteria and to spare the protein-coated viruses. The supernatant was then dropped on fresh lawn culture of the host bacterium in the log phase. After overnight incubation the plaques seen with different morphology were cut and propagated on the host bacterium. The number of phages was increased by inoculating a larger surface area of the host bacterium lawn culture on Roux bottles. The sufficient volume of the harvest was subjected to membrane dialysis at 4°C with three changes of 25% polyethylene glycol buffer three times. The purified phages were suspended in normal saline to have ready to use phages at the concentration of 10^{12} PFU/mL.

Statistical Analysis

Statistical analysis was done using SPSS trial version 21.0 software. For comparing the mean values among the groups, ANOVA, and between the two groups, Student's *t*-test, have been used, if the data followed the Gaussian distribution. If the data did not follow the normality, the Wilcoxon signed-rank test and the Kruskal-Wallis test were applied. If ANOVA/Kruskal-Wallis tests resulted in significant differences, a post-hoc test (Student-Newman-Keuls) was used to determine pairwise differences. The critical value of *p* indicating the probability of significant difference was taken as <0.05 for comparisons at two-tailed tests.

Results

At the beginning of the experiment, none of the stool samples collected from six adult rats yielded *K. pneumoniae*. Table 1 and Figure 1 show that the weight of the pups in all the three study groups was similar at birth. At the end of 7 days, the mean percentage weight gain in groups II and III was 4.8 and 10.1, respectively, compared to group I. The highest percentage of weight gain was observed in the rats fed with *K. pneumoniae* (groups II and III) than the control (group I) during the sixth and seventh weeks (55.7–62.9%, respectively). At the end of the 10th week, the overall percentage of weight gain was 18.4 in group II and 13.3% in group III. The mean percentage weight gain (18.4) was significantly higher in rats fed with *K. pneumoniae* (group I vs group II; $p < 0.021$).

Interestingly when the bacteriophage cocktail therapy was started at the end of 10th week and continued for 15 days, the mean weight of the intervention group III decreased (220.7 g) and was comparable ($p = 0.772$) with the mean weight of the control group (229 g). *K. pneumoniae* could not be isolated from the stool samples after 1 week of the phage therapy. However, the phage therapy was continued for 8 weeks, and comparable weight could be seen in both groups I and III. Interestingly *K. pneumoniae* was observed continuously getting excreted by all the rats of group II. At the end of 30th week, the mean weight in the control group was 228 g, while those on *K. pneumoniae* were 288 g, which was 26.3% higher ($p < 0.029$). The mean weight of group III was 2.8% less at the end of 30th week. However, the mean weight difference between groups I and III was statistically comparable ($p > 0.5$).

Table 1: Percentage gain/loss in rats on oral *K. pneumoniae* and its bacteriophages as compared to the control group of rats

Weeks	*Group I (Control group)*	*Group II (K. pneumoniae + phage therapy at 30th week)*	*Group I vs group II (% weight gain/ loss)*	*Group III (K. pneumoniae + phage therapy at 10th week + repeat K. pneumoniae at 30th week)*	*Group I vs group III (% weight gain/loss)*
0	6	6	00	6	00
1	16.8	17.6	+4.8	18.5	+10.1
2	26.2	25	−4.5	25	−4.5
3	29.4	41.9	+42.5	42.25	+43.7
4	51.8	72.8	+40.5	73.25	+41.4
5	69.4	99	+42.6	108.75	+56.7
6	87.6	136.4	+55.7	115.75	+62.9
7	97.6	151.8	+55.7	142.75	+62.9
8	132.8	165.8	+24.8	156.5	+29.1
9	142.8	180.2	+15.2	171.5	+15.8
10	161.2	190.8	+18.4	182.75	+13.3
11	168.8	209	+23.8	174.25	+9.9
12	182.4	222.6	+22.03	180.75	−0.9
13	193.6	237.6	+22.4	181.5	−6.1
14	194.4	241	+23.9	187.5	−3.5
15	197.2	255.6	+29.6	192	−2.6
16	207.8	248	+19.3	196.75	−5.3
17	207.4	251.8	+21.1	200.25	−3.4
18	207.4	253.4	+22.2	205.75	−0.9
19	207.8	265.6	+27.8	219.25	+5.5
20	206.8	271.6	+31.3	213.25	+3.1
21	202	275.2	+36.2	217.75	+7.8
22	201.2	275.8	+37.1	223.5	+11.1
23	211.2	279.2	+32.2	220.25	+4.3
24	212.6	279	+32	216.5	+1.8
25	213.2	282	+32.3	221	+3.6
26	215.6	285.6	+32.4	215	−0.09
27	226	279	+23.4	223	−1.3
28	229	294	+22.1	226.75	−0.9
30	228	288	+26.3	221.5	−2.8
32	227.4	280	+23.1	219.75	−3.3
34	230.8	259.25	+8.0	219.75	−4.8
36	206	258.5	+25.5	201.5	−2.1
38	222.4	208.5	−6.2	154.4	−30.6
40	233	213	−8.5	280	+20
42	230	223	−3.0	283	+23
44	232.5	225.5	−3	268.5	+15.5
46	235.2	213.75	−9.1	280	+19.1

We did a reversal of therapy at the end of the 30th week. We administered bacteria to the rats on phage therapy (group III) and initiated phage therapy to those who were exclusively on bacteria (group II) up to 10 weeks; we observed a weight gain by 20% in group III in comparison to group I at the end of 38th week. However, group II, which was on phage therapy, lost weight and become comparable to the control group ($p = 0.168$) by the end of 8 weeks (Table 1 and Fig. 1). The percentage of weight gain in group III was observed to be 19.1. Contrary to this, group II had a weight loss of 9.1% compared to the control group I (Table 1). The other significant

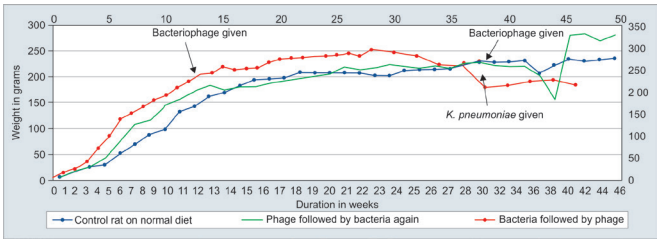

Fig. 1: Weight pattern of rats fed with *K. pneumoniae* and later treated with specific bacteriophage

observation was that one rat in group II died at the age of 32 weeks, weighing exceptionally high, i.e., 375 g, while the average weight of the rest of the rats in group II was 260 g at 30th week.

The levels of cholesterol, LDL, and SGOT were the highest in group I. In group II, the highest level of SGOT, total bilirubin, and direct bilirubin was observed. Further, group II also had the best ratio of HDL/CHOL and TG/HDL. The HDL level in group II was significantly higher than group III (p <0.029). The VLDL, however, was significantly higher in group III than group II (p <0.003). Further, the highest levels of TG and VLDL were observed in group III. Interestingly, the worst ratio between TG/CHOL was also observed in group II (Table 2).

Discussion

In this study, we observed that *K. pneumoniae* contributed to excess weight gain by rats made to colonize the bacterium in their gut. When the oral bacteriophage therapy eradicated the bacterial colonization of 10 and 30 weeks duration in rats, the weight loss could be observed on both occasions. The further surprising observation was that when the bacteriophage treated group of rats were again fed with fed *K. pneumoniae*, they gained a significantly higher weight (p <0.5) than the control group in the next 16 weeks.

Reports indicate that bacteria may produce several metabolites that may affect the composition of the gut microbiota. The metabolites may enter the blood circulation and act on distant organs like the liver, the adipose depot, and even the brain.[24] Pieces of evidence are available suggesting that certain bacteria do colonize the gut of obese persons, which may breakdown the food substrates (polysaccharides). These polysaccharides are usually undigestible by the flora available in lean subjects. These bacteria, if inhabited, may provide up to 40% more calories to the host for absorption and assimilation. Saprophytes can survive on simple carbon sources, e.g., citrate, nitrate, indigestible complex polysaccharides, etc. These saprophytes may produce various short-chain fatty acids (SCFA) like acetate, butyrate, and propionate as well as other metabolites (leptins, leptin receptor inhibitors, other neurotransmitters, trimethylamine, indole etc.),[25] which may help in lipogenesis. These metabolites and neurotransmitters may stimulate the hunger center. The SCFA like acetate may lead to fat deposition. Acetate is known for its potential for increased lipogenesis.[26]

Further, these bacteria may also cause auto-brewery syndrome because of their high fermentative abilities leading to the adverse effect of alcoholism, apart from obesity.[22] The authors have stated that alcohol drinkers following weekly low-risk drinking guidelines are not insulated from harm.[27]

We have to consider the present data with caution that *K. pneumoniae* may not be the only bacterium involved in excess weight gain. This is quite possible that other bacteria of saprophytic nature with good fermenting activity can cause more energy harvesting in the gut. The possible mechanism might be that these saprophytes can utilize even the citrate like simple carbon substrates. In support of this speculation, the plant *Garcinia* extract, hydroxy-citric acid, has been found to cause weight loss by competitively inhibiting the enzyme adenosine triphosphatase (ATP)-citrate-lyase.[28–31] Cytosolic acetyl-CoA synthesized by ATP citrate lyase is the primary enzyme responsible in many tissues. Cytosolic acetyl-CoA is used in several critical biosynthetic pathways, including lipogenesis and cholesterologenesis.[32] *K. pneumoniae* can utilize the citrate through a fermentative pathway involving carrier CitS, citrate lyase, oxaloacetate decarboxylase.[33] *K. pneumoniae* is also known for bioconversion of pentose sugars of hemicelluloses to ethanol.[34] Therefore, it is worth looking for the colonization of alcohol-producing bacteria in the human gut, which might cause all the adverse effect of alcohol consumption including weight gain despite being a teetotaler. The significance of the present work is that if a bacterial association is established with obesity; the culprit bacteria may be eradicated using specific bacteriophages.

Table 2: Showing lipid profile and liver function tests in different groups

	Group I (mean ± SEM)	*Group II (mean ± SEM)*	*Group III (mean ± SEM)*	*Significance*
Cholesterol (mg/dL)	109.40 ± 29.11	87.80 ± 32.51	91 ± 17.31	
TG (mg/dL)	216.40 ± 50.25	137.60 ± 74.40	307.25 ± 71.66	2 vs 3, p <0.003
HDL (mg/dL)	41.80 ± 4.87	37.00 ± 6.44	46.00 ± 4.24	
LDL (mg/dL)	24.32 ± 35.77	23.28 ± 31.13	16.20 ± 19.73	
VLDL (mg/dL)	43.28 ± 10.05	27.52 ± 14.88	61.45 ± 14.33	2 vs 3, p <0.003
SGOT	239.80 ± 73.67	162.20 ± .20.33	138.75 ± 33.95	
SGPT	101.00 ± 118.32	112.60 ± 98.50	35.25 ± 8.06	
Total bilirubin	0.34 ± 0.05	0.44 ± 0.22	0.38 ± 0.10	
Direct bilirubin	0.10 ± 00	0.12 ± 0.04	0.10 ± 0.00	
TG/HDL				1 vs 2, p <0.012
HDL/VLDL				2 vs 3, p <0.033
LDL/VLDL				2 vs 3, p <0.038

TG, triglycerides; HDL, high-density lipoprotein; LDL, low-density lipoprotein; VLDL, very low-density lipoprotein; SGOT, serum glutamic-oxaloacetic transaminase, SGPT, serum glutamic pyruvic transaminase

The implication of the detection of the highest HDL level, the highest HDL/CHOL, and the lowest TG/HDL ratios in the rats on bacterial therapy needs explanation. However, the highest SGPT, along with elevated total bilirubin and direct bilirubin (an indicator of liver damage), indicates that prolonged colonization with *K. pneumoniae* may cause injury to liver parenchyma. In support of this statement, *E. cloacae* have been reported in inducing hepatic damage and subcutaneous fat accumulation in mice on a high-fat diet.[35]

In conclusion, significant advances have been made, and our understanding concerning obesity is improving. The data presented in this study are based on a minimal number of rats. The high SGPT in group II might be indicating liver damage. Contrary to this, the excellent ratio between HDL/CHOL and HDL/TG in group II may indicate that *K. pneumoniae* may help maintain these healthy ratios. The highest levels of TG and VLDL in group III remain to be explained. Therefore the experiment may be repeated with many more animals to have robust data about the relationship between *K. pneumoniae* colonization and obesity and also its amelioration by specific bacteriophages can be verified. Exclusive dietary foods are usually associated with unpredictable outcomes. Therefore, the ultimate aim is to develop a personalized intervention if the causative agent/s or factors are known. Specific bacteriophage therapy may be a significant modality in this direction. There is an *in vitro* study where feces treated with specific bacteriophage before transplantation prevented the development of nonalcoholic fatty liver disease.[22] Our study is unique as we have fed the rats with the bacteria and eradicated it with a particular cocktail of phages, establishing the role of *K. pneumoniae* in obesity. However, a lot more is required to delineate the relationship between obesity and microbes and specific bacteriophage therapy.[36]

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