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RESEARCH ARTICLE

Molecular Detection of Some Virulence Genes of Klebsiella pneumonia Isolated from Camels

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ABSTRACT

Klebsiella pneumonia is internationally recognized as one of the most important microorganisms that causes defect in economic importance to the dairy industry, affecting almost all domesticated species of animals and being reported from all over the world. This study aimed to detect K. pneumonia isolated from Camels and studying some serious virulence factors of k. pneumonia in comparison with those isolated from different animals. Sixty nasal and fecal samples were collected from camels. Many isolates on MacConkey were in different colors on chrome orientation media, the results on this differential media showed primary identification of 55 % Klebsiella, 21 % E. coli, 15 % Enterococcus, and 9 % Pseudomonas. However, the quantitative PCR for (16S rRNA) confirmed (82%) of total suspected isolates were K. pneumonia. Multi-sequences alignment of this sequenced isolate showed a high identical score to K. pneumonia strains from Hong Kong and Nigeria. The results also showed 100% of K. pneumonia isolates were resistant to Vancomycin and highly sensitive toward the others like Levofloxacin, Trimethoprim, and Ceftriaxone. The molecular detection results for some virulence factors genes (ESBL) showed variable differences of these genes in comparison with recently reported researches on bovine such as (90% CTX, 100% INT1, 60% INT2, 60% KPC, 10% HEMO and 0% of SHV). It was concluded that the livestock and body immune of camels that less exposure to antibiotics plays an important role for restraining the development of the resistance systems of K. pneumonia towards antibiotics.

INTRODUCTION

There are differences in digestive systems of camels, cattle and sheep. Although, most true ruminants own four chambered stomach, the stomach of camels has only three chambers without omasum (1).

Many pathogens can be harbored within the gastrointestinal tract of food-producing animals (2). Contamination of meat during processing is a rational expectation. *K. pneumoniae* is opportunistic bacteria that exist commonly in the gastrointestinal tracts of animals (3). It is a member of Enterobacteriaceae that was observed as a significant pathogen responsible for equally acquired nosocomial and public infections (4). It is also connected with the formation of extended-spectrum β – lactamases (ESBL), which belong to the CTX–M and SHV relatives (5). ESBLs have been described

as a part of complex integrons, which facilitate their horizontal transfer to other related and unrelated, microbes (6).

Integrons are conserved DNA sequences carried on episomal genetics structures that deliver a crucial approach for capturing and dispersion the genes of antimicrobial resistance (7). Two conserved regions are involved within Integrons that are neighboring variable regions holding single or multiple resistance genes (8). Detailed descriptions of their structure appeared as three genetic units constructed from integrase gene, gene cassettes (att), and a site of integration. Due to the integrons are immobile, the gene cassettes will be excised and integrated by the integrase in order to form the integron (9). In spite of their contribution gene traffic, they also can be located within transposons or conjugative plasmids, as well as, bacteria can acquire new genetic meterial (10). The most predominant genes are class 1 integrons, which found in ESBL frequently, these are commonly produced in medical bacteria of Enterobacteriaceae such as *K. pneumoniae*. Less frequently class 2 Integrons could also be present in ESBL of *E. coli* and *K. pneumoniae*. In contrast, the production of class 3 Integrons are very rare although they are o found in ESBL (11).

The cytolysis toxin (Hemolysin) is produced by some microorganisms, which possess these virulence factors and act on erythrocytes for lysis. This is associated with the pathogenesis of these microorganisms (12,47). Hemolysins are significant causes of host damage due to facilitating bacteria dissemination. It also may cause alteration in host pathways, cell survival, cytoskeletal dynamics, and inflammatory response, (13). In 2001, the KPC-type enzyme was first described in North Carolina from *K. pneumoniae* strains that resist carbapenem (14). This enzyme was classified later as one of Ambler class A enzymes (NMCA, IMI, SME, GES, and KPC), which are acquired carbapenem-hydrolyzing β-lactamases (15). Although, the description of this enzyme participation activates the process of spreading, the information of rapid spread and the responsible genetic elements is quite few (16). There is association between The KPC gene (*Klebsiella pneumoniae* carbapenemase and the plasmid-borne transposon Tn*4401* that may cause the rapid dissemination (17,46).

The aim of our study is to detect *K. pneumoniae* in camels in Al-Muthanna province desert /Iraq, and examine their resistance to several antibiotics. In addition to detect some of the virulence genes in these bacteria

METHODOLOGY

Sixty nasal and fecal swabs were collected from camels suffering from respiratory infection (heavy nasal discharge and lacrimation), as well as signs of mild diarrhea. These camels were herded in the desert of Al-Muthanna province/ Iraq. This study was settled from November 2021 to February 2022. The samples were transported to the laboratory using sterile tools and transport media tubes for appropriate examination. Filled swabs of all samples were used for culturing on MacConkey agar, which is more specific for gram-negative bacteria, that was prepared based on manufacturer instructions and incubated 18 hrs at 37 ºC (18). All isolates were then cultured on Chrome orientation media and incubated 18 hrs at 37 ºC, which is a differentiated media for some bacteria based on the morphological description of colony and media color change (19).

DNA Extraction

Colony of suspected *K. pneumoniae* isolates were inoculated for 16 hours in nutrient broth. Genomic DNA extraction from 1 ml overnight growth samples was done using manufacturer instructions (Qiagene Kit). The extracted DNA was examined by Nanodrop spectrophotometer and then store at -20 °C at freezer until usage. (20,45)

Bacteria detection by PCR reaction

Gradient PCR technique was carried out on all isolates for more detection of bacteria, all PCR samples were prepared as per manufacturer instructions (1.5 µl of 10 pmol forward and reverse primers, 5µg

genomic DNA, 12.5 Mastermix, and complete volume to 25 µl of nuclease-free water. Primers for 16S rRNA were used to confirm the presence of *K. pneumoniae* as shown in table (1). PCR programs were set according to the annealing temperature of each primer (95 °C for 5 minutes, for 30 cycles of 94 °C for 30 seconds, 58 °C for 30 seconds, 72 °C for 45 seconds, and a final extension at 72 °C for 7 minutes, with a final hold at 4 °C (Thermo cycler; fisher, Germany). PCR products were then loaded on 1.5% agarose gel electrophoresis using 1X Tris-Borate- EDTA (TBE buffer), which was prepared by mixing Tris base 10.8 g (89 mM), boric acid 5.5 g (2 mM), 4 mL of 0.5M EDTA (pH 8.0), and then the components were dissolved to 1 L of distilled H2O .and run for 60 min/ 80 volts (21). DNA bands were stained with ethidium bromide that was measured by a 1500 bp DNA ladder to confirm the specific size of 16S rRNA genes for the subjected bacteria. UV Transilluminator for DNA detection was used for imaging.

Table 1: Primers of 16S rRNA gene

Bacteria		Primer sequence	Tm	Amplicon	REF
K. pneumoniae	F	AGAGTTTGATCCTGGCTCAG	60	1500bp	(22)
	R	GGTTACCTTGTTACGACTT			

Partial sequencing of 16S rRNA gene

A purified sample (40 µl) of 16S rRNA PCR product was prepared and sent out to Macrogen Company (Korea) for sequencing. NCBI BLASTn engine was carried out for indicating the presence of remarkable homology with the expected target that cover a maximum portion of 16S rRNA gene within *K. pneumoniae* genome sequences, then the sequenced data was applied for multiple alignments using MEGA X software (Version: MEGA, 11.0.11) in order to construct a phylogenetic tree to discover the genetic variation between the subject strain with global strains of *K. pneumoniae*.

Antibiotic sensitivity test

Muller Hinton agar was prepared following manufacturer instructions for examining the Antibiotics sensitivity of *K. pneumoniae*. Isolates were streaked on plate by a cotton swaps, then ten different types of antibiotics discs (HIMedia manufacturer) like (Amikacin 10µg, Trimethoprim 10µg, Amoxicillin 25µg, Ceftriaxone 10µg, Levofloxacin 5µg , Vancomycin 30µg, Tetracycline 10µg, Nitrofurantion 100µg, Cefixime 5µg, Clarithromycin 5µg) were distributed on two plates, five different antibiotics on each plate. The incubation process was carried out for 16 hours at 35º C (25).

Virulence factor detection

Based on the results of Antibiotic sensitivity, *Klebsiella* isolates were used for the detection of virulent genes by gradient PCR in order to find the variation of these genes in comparison with the same bacteria in different animals. Primers of six different genes were designed for the molecular detection table (2). PCR samples and settings were as mentioned above. All PCR products were also loaded on 1% agarose gel and run for 40 min/ 80 volts (26), UV Transilluminator was used for DNA checking.

Table 2: Primers of virulence factors genes of K. pneumonia

Gene		Primer sequence	Tm	Amplicon	REF.
SHV	F	ATGCGTTATATTCGCCTGTG	55	730 bp	(26)
	R	TGCTTTGTTATTCGGCCAA			
CTX	F	CGCTTTGCGATGTGCAG	55	550 bp	(27)
	R	ACCGCGATATCGTTGGT			
INTO1	F	CAGTGGACATAAGCCTGTTC	55	160 bp	(28)
	R	CCCGAGGCATAGACTGTA			
INTO2	F	CAGGGATATGCGACAAAAAGG	54	788 bp	(29)

	R	GTAGCAAACGAGTGACGAAATG			
KPC	F	GCTACACCTAGCTCCACCTTC	55	989 bp	(30)
	R	ACAGTGGTTGGTAATCCATGC			
HEMO	F	CCGGAGCGTTTTTCGATTGG	57	413 bp	(31)
	R	AGCATCCGGGTAAAAAGGGG			

RESULTS

Isolation and identification

All samples were cultured on MacConkey. The results showed various morphology of colonies based on color and lactose fermentation, they were pink mucoid and fermented, pink dry and fermented, brown non-fermented, and gray non-fermented. On the other hand, the growth on Orientation Chrome media after taking a single colony from MacConkey culture gave an initial indication of the type of bacteria according to morphological characteristics (Figure 1). They were 55 % Klebsiella, 21 % E. coli, 15 % Enterococcus, and 9 % Pseudomonas. Table 3.

Table 3: characteristics of bacteria on Chrome orientation medium

Bacteria	MacConkey	Orientation Chrome agar	Number of Isolates	%
Klebsiella	pink mucoid and lactose fermented	Metallic to dark blue color	33	55
E. coli	pink dry and lactose fermented	Dark rose to pink	13	21
Enterococcus	brown non lactose fermented	Turquoise to green	9	15
Pseudomonas	gray non lactose fermented	Creamy to transparent	5	9

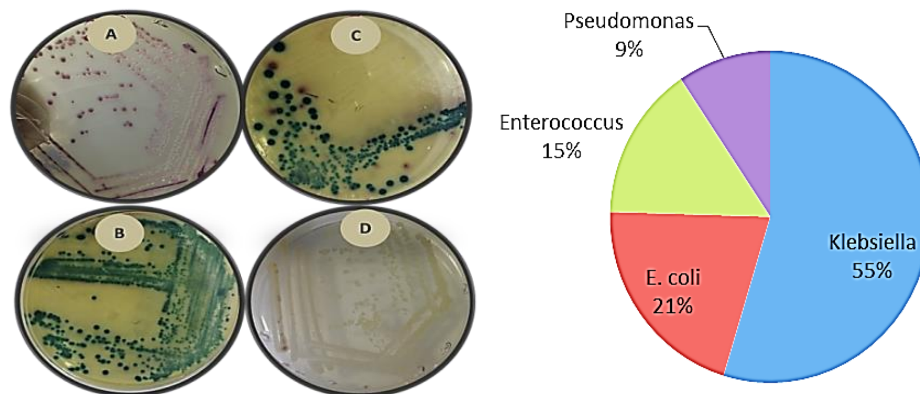

Figure 1: Morphological analysis on orientation Chrome agar. Images refer to:

A- E. Coli, Pink colonies
B- Enterococcus, Green colonies
C- Klebsiella, Metallic Blue colonies
D- Pseudomonas, creamy color

Identification of bacteria -16S rRNA

According to the estimated results of Orientation Chrome agar, the suspected *K. pneumoniae* isolates were determined by PCR technique using specific primers with (1500 bp) product sizes Table 1. The PCR results on agarose gel were recorded (82%) of the total suspected isolates for *K. pneumoniae*, (Figure 2).

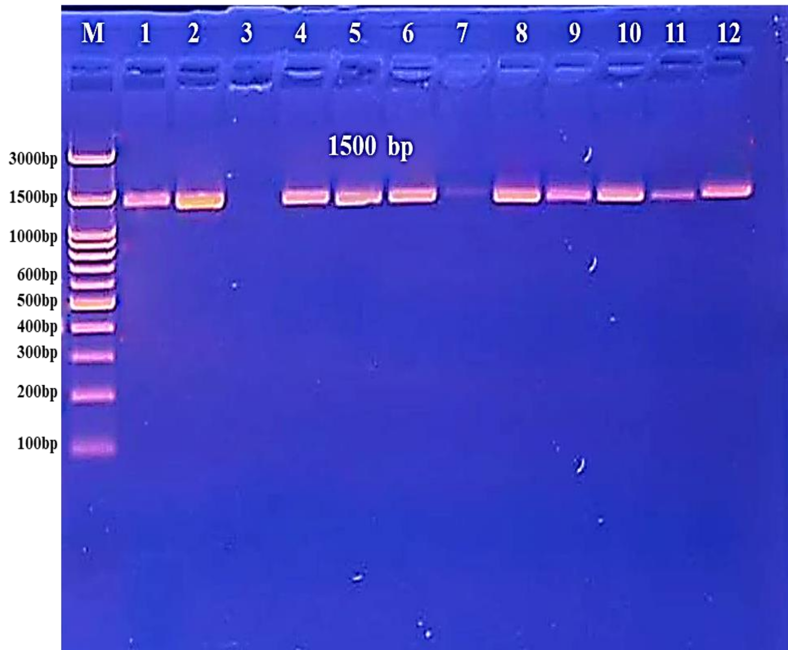

Figure 2: 16s rRNA gene detection of bacteria using PCR technique

It shows PCR products of the subjected bacteria with particular sizes on agarose gel. (M) 3 Kbp DNA marker according to the size of amplicon. (1) Positive control, (2) Negative control, (3-10) positive results of *K. pneumonia.*

Phylogenic analysis

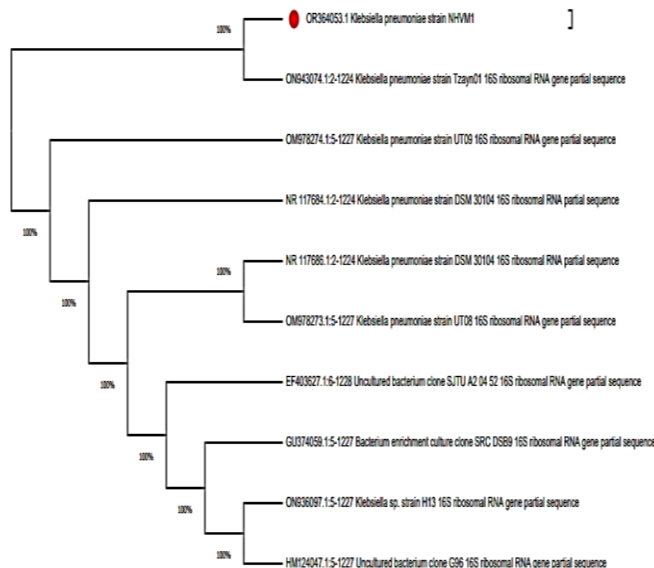

Figure 3: it is the phylogenetic tree diagram that shows the similarity approach between the new strain (NHVM1, yellow highlight) and other closely related global strains of K. pneumoniae according to the sequence of 16S rRNA gene. The tree was

constructed based on the neighbor-joining method. Numbers at nodes represent levels of bootstrap support (%) based on analysis of 1000 replications.

Antibiotics sensitivity

The results showed that the *K. pneumoniae* isolates were highly sensitive for most of subjected

ntibiotics based on standard measurements, especially, Levofloxacin, Trimethoprim, and Ceftriaxone, which gave a large size of inhibition zone (over 30 mm) Figure 4. Meanwhile, the resistance was noticeable for Vancomycine (100%) and Amoxicillin (75%).

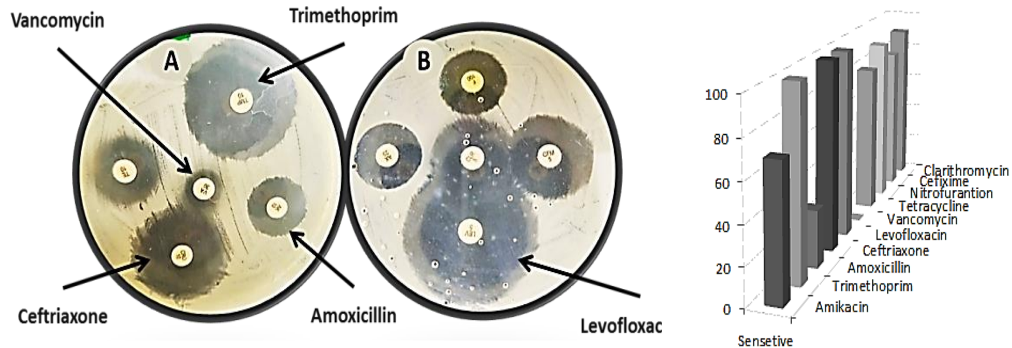

Figure 3: Antibiotic sensitivity test
It shows the resistance ratio of Klebsiella towrds Vancomycin and Amoxicillin. Although its sensitivity towards the rest of applied antibiotics. The efficiency of Levofloxacin, Trimethoprim and Ceftriaxone on this bacteria was very opvious as zone of inhibtion showed

Detection of virulence genes

Twenty-seven *K. pneumoniae* isolates were then examined by PCR technique in order to check the presence of some virulence factors by utilizing of specifically designed primers with various sizes of PCR products (Table 2). The results showed the presence of (100, 60) % Intgron genes 1&2 respectively, 90% CTX gene. 60% KPC gene, and 10% of Hemolysis gene. Meanwhile, SHV was not detected during this assay (Figure 5).

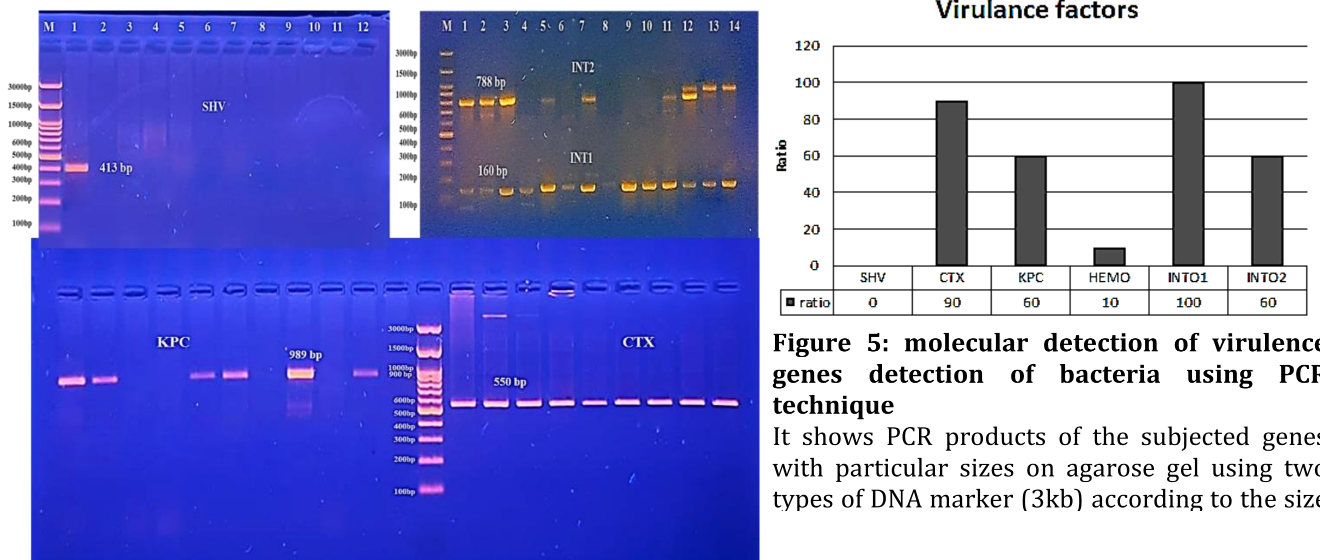

Figure 5: molecular detection of virulence genes detection of bacteria using PCR technique
It shows PCR products of the subjected genes with particular sizes on agarose gel using two types of DNA marker (3kb) according to the size

DISCUSSION

Gram-negative bacteria like *K. pneumoniae* are a leading cause of nosocomial infections such as UTI, It generally exists in the alimentary tract of human and animal (32). Although the recent study showed the big role that the microbiota of bacteria plays against upgrading cancer (33). The critical point of *K. pneumoniae* infection is that late diagnosis or undiagnosed will significantly lead to renal failure. In this study, 55% of total fecal samples were determined as *K. pneumoniae* based on the growth on Orientation Chrome media. Although this media has a deferential function and helps to obtain a probable identification, it still cannot give 100 % guarantee to detect bacteria. This uncertainty could depend on the genetic features and the source of the isolates (19).

Subsequently, the particular detection was confirmed by quantity PCR technique, which confirmed that 82% of the total samples were *K. pneumoniae*. These kinds of differences might follow many reasons, firstly, Colony morphology and biochemical features on Chrome agar could be similar to other bacteria, and the subjected bacteria might obtain critical genetic mutations that made 16S rRNA gene difficult to be recognized by their specific primes. Moreover, a technical issue during DNA extraction or PCR sample preparation stages (34).

The current project identified isolate that highly similar in nucleotide sequences to isolates from Hong Knog, Nigeria, China, and India. This similarity with those from these countries could be due to an evolutionary manner that created new K. pneumoniae strains in Iraqi camels. As well as, the possibility of presence unexplored reservoir that transported this opportunistic pathogen world widely. Moreover, the antimicrobial resistance (AMR)-encoding genes that are carried naturally on several plasmids for expressing of MDR phenotypes in these bacteria consider a main challenge to human health, animal, and environment (35).

During the Antibiotics sensitivity test, *K. pneumoniae* isolates were highly sensitive to Levofloxacin, Trimethoprim, and Ceftriaxone. Meanwhile, they were resistant to Vancomycin (100%) and Amoxicillin (75%). These results were in contravention with what Molana (32) verified that K. pneumonia strains isolated from clinical samples in Babol city were resistant to cefotaxime (90%), ceftriaxone (60%), and imipenem (60%) (36), this might be due to wildlife of camels with a good immune system and less exposed to antibiotics made K. pneumoniae resistance system undeveloped in comparison to those isolated from human. On the other hand, the inactivation of β-lactam drugs like monobactram and cephalosporin (19), especially The ESBL bacteria increased and led to treatment failure with healthcare fee problems (37). Studying bacterial resistance genes to β-lactamase is required. CTX and SHV genes of Positive ESBLs are the main elements of bacterial drug resistance to β-lactam antibiotics (38). In this study, the detection percentage of CTX-M ESBLs in K. pneumoniae in camels was (90%) which is less than what Al-Dabbagh (39) recorded (100%) and higher than the observed results in Iran and Beijing human isolates (78.9%), (84.80%), respectively (39). This refers to the regional impact of increasing the use of antibiotics. Contrariwise, the current study reported zero percentages of SHV ESBL-producing organisms; this was incompatible with Indian and Korean studies which reported 6% - 87% frequency of ESBL-producing *K. pneumoniae*. (40, 41). SHV class of enzymes is the progenitor *K. pneumoniae* and up to 20% of the plasmid-mediated ampicillin resistance under its control. Additionally, SHV-1 β-lactamase gene might develop as a plasmid or chromosomal gene in *K. pneumoniae* (42). The results also showed no detection of the Hemolysis gene (hly) in all *K. pneumoniae* isolates which is exactly what Kuş mentioned in their study (43). Moreover, the rate of KPC gene detection in this study was (60%), which was a noticeably lower rate than what Robledo (43) revealed (73%) (44). Altogether of *K. pneumoniae* isolates were positive for integron class 1. This was as same as Asghari (42) study confirmed. The high rate of integron class 1 might be due to the relationship between integron I and the occurrence of multi-drug resistant gram-negative bacteria (43). Meanwhile, the prevalence of class 2 integrons in our MDR K. pneumoniae isolates was 60%, which is higher than that termed by Asghari (42)., and Firoozeh (44).

CONCLUSION

Overall, we can conclude that the noticeable variation in the rates of development of antibiotic resistant and virulence systems of isolated *K. pneumoniae* relative to body immune and livestock of camels, which are apparently less exposing to have antibiotics during illness in comparison to another domestic animal.

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