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Antibiotic Resistance Patterns in Faecal *E. coli*: A Longitudinal Cohort-Control Study of Hospitalized Horses

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1. Introduction

Cross-sectional prospective surveys are a useful method for studying the effects of antimicrobials on animals (Dunowska et al., 2006; Thomson et al., 2008; Bunner et al., 2007) . However, there is a paucity of these studies in horses compared to other animals (Coe et al., 2008).

Although antibiotic consumption has been a major contributor to the antibiotic resistance phenomenon (Bunner et al., 2007) various different factors have added to the development and dissemination of antimicrobial resistance. For example, population densities among humans have been identified as risk factors for development and spread of antimicrobial resistance (Bruinsma et al., 2005; Zhang et al., 2006). Hospitalization, in humans for instance, is also associated with an increase in antibiotic resistance in pathogenic bacteria, while others have found a lack of a significant effect on the prevalence of resistance in *E. coli* (Koterba et al., 1986; Gaynes et al., 1997). A study by Dunowska and colleagues (Dunowska et al., 2006) concluded that both antimicrobial administration and hospitalization were associated with the shedding of *E. coli* resistant strains from equine faecal samples.

Certain antimicrobial administration regimes have been shown to give rise to antibiotic resistant bacteria, which then comprise a reservoir of resistant bacteria when shed into the environment (Ahmed et al., 2010; Fofana et al., 2006; Diarrassouba et al., 2007; Pallecchi et al., 2007). Linked resistance genes encoded on mobile genetic elements, can also contribute to the spread of resistance genes (Srinivasan et al., 2007), with exposure to one antimicrobial

agent leading to selection for resistance against other, or multiple, antimicrobial drugs (Braoudaki et al., 2007; Schnellmann et al., 2006; Weese et al., 2006). Such genes can be maintained after antibiotic treatment, has been stopped such that removing the selective pressure does not necessarily lead to the loss of resistance (Ahmed et al., 2010; Kaszanyitzky et al., 2007; Ghidan et al.,2008). Mobile genetic elements are widely reported cause of the spread of antibiotic resistance in both *E. coli* and *Salmonella* commensals in animals bred for human consumption (Roest et al., 2007). Therefore, *E. coli* and other enteric organisms are widely used as an indicator organism (Kaneene et al., 2007; Bruinsma et al., 2003).

The purpose of this investigation was to identify changes in antibiotic resistant *E. coli* in faeces of horses entering the Philip Leverhulme Equine Hospital (PLEH), at the University of Liverpool, UK on arrival, during hospitalization, and after discharge. The dynamics affecting the prevalence of antibiotic resistant *E. coli* were used in this study in order to examine potential risk factors.

2. Materials and methods

2.1 Study design

Faecal samples were collected from horses admitted to the Philip Leverhulme Equine Hospital (PLEH) at the University of Liverpool for more than seven days at the following time points: 1st, on arrival, before treatment began; 2nd, one day; 3rd, 2-3 days after treatment had started; 4th, immediately before discharge. Further faecal samples were collected by the horse's owners, 4-8 weeks after discharge (5th), and also 6 months after discharge (6th). Horses were divided into three groups as follows: GI+, horses with gastrointestinal conditions and under antibiotic therapy; Non-GI+, horses with non-gastrointestinal conditions and under antibiotic therapy; Non-GI-, horses with non-gastrointestinal conditions and no antibiotic therapy.

2.2 Sample collection

Faecal samples were taken from stalls randomly and chosen from the firm part of the faecal balls. In total, 2-3 grams were collected and taken straight to the laboratory.

2.3 Bacterial culture

Standard microbiological methods and biochemical tests were used to isolate and confirm each *E. coli* as fully described by Ahmed et al 2010. Three single *E. coli* colonies were chosen from each sample, confirmed by biochemical testing (e.g. API system) and subjected to further susceptibility tests thereafter.

2.4 Antibiotic susceptibility tests

Antibiotic susceptibility testing was performed according to the BSAC guidelines (Andrews, 2008). Briefly, antimicrobial drugs tested for included: - ampicillin; if the isolates show resistance to ampicillin then isolates were also tested against other two cephalosporins (cefotaxime (30 µg) and ceftazidime (30µg) for extended resistance to cephalosporines and referred as potential ESBL producers (ESBLs*) for ampicillin resistant isolates), apramycin, chloramphenicol (and also against florfenicol, if chloramphenicol resistant), nalidixic acid

(and also against ciprofloxacin, if nalidixic acid resistant), tetracycline and trimethoprim. Further susceptibility tests were also performed for gentamicin, spectinomycin, streptomycin and sulphamexazole for all collected resistant isolates. Isolates were considered resistant if resistance to at least one antibiotic was shown and classified as multidrug resistant isolates (MDR) if resistant to four or more classes of antibiotics (Ahmed et al., 2010). Guidelines for determining florfenicol and apramycin resistance were as followed and determined by Ahmed et al., 2010.

2.5 Identification of antibiotic resistance genes in resistant E. coli isolates

DNA was extracted by boiling: a 5µl drop of each isolate was suspended in 0.5 ml sterile water and heated for 20 minutes at 100°C. PCR assays, previously applied by Ahmed et al 2010 were also used to detect genes commonly associated with ampicillin, chloramphenicol, tetracycline and trimethoprim resistance, were carried out using modified versions of published protocols: Pitout, 1998 (Pitout et al., 1998) for ampicillin resistant genes (*tem* & *shv* genes); Vassort-Bruneau, 1996 (Vassort-Bruneau et al., 1996) and Keyes et al.,2000 (Keyes et al., 2000) for chloramphenicol resistant genes (*catI, catII catIII* & *cmlA* genes); Ng, 2001 (Ng et al., 2001) for tetracycline resistant genes (*tetA, tetB, tetC, tetD, tet E* and *tetG* genes); Gibreel & Sköld, 1998 (Gibreel et al., 1998) and Lee, 2001 (Lee et al., 2001) for trimethoprim resistant genes (*dfr1*, *dfr9 dfr12, dfr13, dfrA14* & *dfr17* genes).

2.6 Statistical data analysis

Data were analysed using Minitab software, in order to determine the 95% binomial confidence intervals (95%CI) and chi-square test (X^2).

2.7 Conjugation assays

Mating experiments to determine if resistance could be transferred by conjugation were carried out using a nalidixic acid resistant *E. coli* K12 as the recipient (as performed by Ahmed et al., 2010). The method was as following: *E. coli* K12 was inoculated into 20ml nutrient broths (LabM) and incubated overnight at 37°C. Resistant *E. coli* strains (donor strains) were inoculated into separate 3ml nutrient broths and incubated overnight; 4 ml of recipient strain was then added to the donor strain and incubated at 37°C for one hour. Broths were then streaked onto agar plates containing nalidixic acid (30µg/ml) plus ampicillin (8µg/ml). Plates were incubated for 24 hours. Successful transconjugants were subcultured onto nutrient agar for susceptibility testing by disc diffusion as previously described. The resistance profiles of the transconjugants were compared to the resistance profile of the original strains. Gene profiles of the donor isolates, characterized by PCR, were described prior to the tranconjugation experiments

3. Results

3.1 Prevalence of antibiotic resistant (AR) *E. coli* isolate

In total, 15 horses were used for the study: GI+ (n=6 horses), non-GI+ (n=4 horses) and non-GI- (n=5 horses). Six samples were collected from each horse (n=90 in total). The distribution of antibiotic resistance is presented in Tables 1 and 2.

Cohort group	No. of horses	No. of samples collected	No. of samples positive for AR *E. coli* (%)	Distribution of resistant samples(out of possible 6) for each sampling time					
				1st	2nd	3rd	4th	5th	6th
GI +	6	36	21 (58%)	2	4	5	5	3	2
NON GI +	4	24	18 (75%)	3	4	4	4	3	0
NON GI –	5	30	16 (53%)	1	3	4	4	4	0

Table 1. The number of horses, faecal samples collected and faecal samples positive for at least one antibiotic resistant (AR) *E. coli* isolate

Source of samples	Samples collected	Positive samples	Distributing of samples containing *E. coli* resistant isolates to different antibiotics									
			AMP	CEP	APR	CHL	FLO	NAL	CIP	TET	TRI	MDR
GI +	36	21	15	1	1	5	5	8	6	15	19	8
NON GI +	*24*	18	12	10	0	10	0	11	11	11	18	11
NON GI –	*30*	16	14	4	0	7	2	7	5	11	13	10

*Abbreviations: Ampicillin (AMP), Cephalosporins (CEP), Apramycin (APR), Chloramphenicol (CHL), Florfenicol (FLO), Nalidixic acid (NAL), Ciprofloxacin (CIP), Teracycline (TET), Trimethoprim (TRI), MDR (multidrug resistance i.e. resistance to four or more antimicrobials), *ESBLs* isolates show resistance to ampicillin then isolates were also tested against other two cephalosporins (cefotaxime (30 µg) and ceftazidime (30µg) for extended resistance to cephalosporines andreferred as potential ESBL producers (ESBLs*)

Table 2. Summary of horses, faecal samples, faecal samples containing resistant *E. coli*, and the number of faecal samples with *E. coli* resistant to each individual antibiotic

The proportion of samples with at least one *E. coli* isolate resistant to at least one antibiotic ranged from 53-75% but did not vary significantly between treatment groups (GI +, non-GI+, non GI-) (Table1). All three treatment groups also showed a similar change in prevalence of resistant isolates recovered over the duration of the study (Table1). Furthermore, there were no significant differences in the antibiotic resistance profiles of the isolates in each group (Table2). Therefore, data from the three groups were subsequently combined for the analysis of changing resistance over time. A definite pattern was observed in the prevalence of overall resistance, which increased from 40 +/-6 % at the first time point, immediately prior to admission, to 86 +/- 28 % during hospitalization (3rd time point), and decreased to 12 +/- 30 % after release (6th time point) (Figure1).

To compare the prevalence of resistant isolates before hospitalisation, immediately before discharge and 6 months after discharge (at 1st, 4th and 6th time points respectively), data was analysed by X^2 testing, analysing each individual antimicrobial as well as multidrug

resistance. With the exception of ampicillin, isolates resistant to each antimicrobial drug and multidrug resistant isolates (MDR) (i.e. isolates resistant to ≥ 4 antibiotic classes), increased significantly during hospitalization and decreased after the horses had returned home (Table3). The numbers of isolates resistant to florfenicol were too low for statistical analysis.

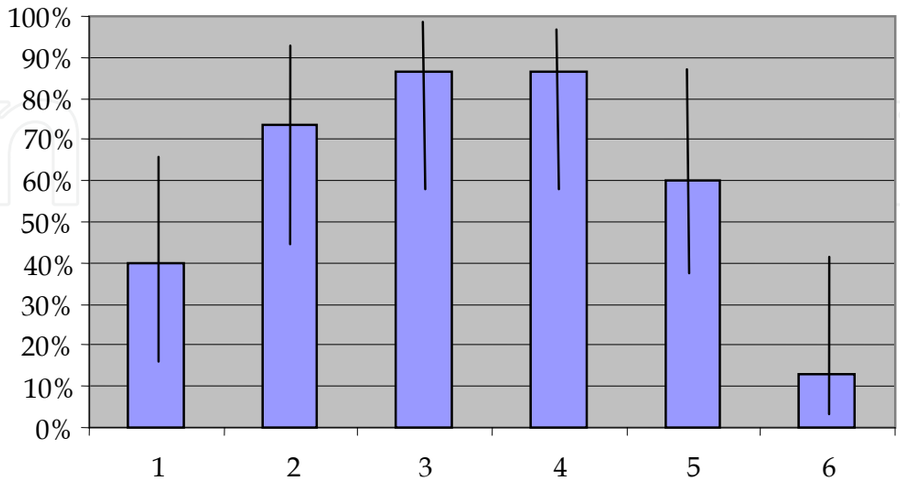

Fig. 1. Relationship between sampling time and the proportion of samples with ≥ one isolate resistant to at least one antibiotic (with 95% binomial CI); total from all three treatment groups GI+, non-GI+ and non-GI are combined (table1).

Prevalence of antibiotic resistance at sampling times	**Sampling times 1 & 4**		**Sampling times 4 & 6**	
	X^2	p	X^2	p
Resistant to at least one antibiotic	7.03	≤ 0.01	13.4	≤ 0.001
Amp	3.394	≥ 0.05	9.600	≤ 0.01
Cep	6.000	≤ 0.05	6.000	≤ 0.05
Chlo	6.136	≤ 0.05	9.130	≤ 0.01
Nal	7.500	≤ 0.01	7.500	≤ 0.01
Cip	7.500	≤ 0.01	7.500	≤ 0.01
Tet	15.000	≤ 0.001	11.627	≤ 0.001
Tri	10.995	≤ 0.001	13.393	≤ 0.001
MDR	10.909	≤ 0.001	10.109	≤ 0.001

Table 3. Two X^2 tests (2x2 analysis) to compare the effect of hospital admission and discharge on prevalence of resistance to particular antibiotic drugs.

3.2 Susceptibility testing of culture collection

In total, 138 *E. coli* isolates resistant to at least one antibiotic were collected. Of these, 71 (51.4%) were classified as MDR. Among these, two main distinctive MDR phenotypes (Ph_s) were found: Ph_s1;Amp,chlo,tet,tri,nal, comprising 93% of the MDR isolates, and mostly found among non-GI + samples; Ph_s2; Amp,chlo,tet,tri comprised 50% of the MDR isolates among the GI+ and the non-GI- samples. All the resistant isolates (n=138) were tested for

susceptibility to further antibiotics. Overall, 38.5% were resistant to gentamicin, 71% to spectinomycin, 96% to streptomycin and 90% to sulphamexazole.

3.3 Molecular analysis of culture collection

PCRs revealed that *CatI [only](87%)*, *tem(60.8%)*, *tetA(60.8%*, *dfr17*(50.4%), *dfr1(38.5%)* were identified at higher prevalence among each positive collection of resistant isolates to each antibiotic (table4). MICs were also shown at higher values of ≥256 for most isolates to the selected drugs (table4).

Antibiotic	No. of Positive Sample	No. of Positive Isolates	MICs (ug/ml)	Resistance genes
Ampicillin	41	95	128 - >256	*tem (60.8%)* *shv (2%)*
Cephalosporines	15	34		
Chloramphenicol	22	51	256 - ≥256	*catI* (only) *(87%)*
Florfenicol	7	7		*catI (71%)*
Nalidixic acid	26	65		
Ciprofloxacin	22	64	4 – 16	
Tetracycline	37	89	64 - ≥256	*tetA (60.8%)* *tetB (19%)* *tetA&tetB(11.9%)*
Trimethoprim	50	127	>256	*dfr17* (50.4%) *dfr1 (38.5%)* *dfr12* (20%)

Table 4. Summary of antibiotic resistance, showing levels of resistance, MIC values and resistance gene prevalence in a total of 90 faecal samples and 138 isolates.

3.4 Conjugation experiments

Mating experiments were performed on selected isolates (n=73); those exhibiting nalidixic acid resistance were excluded 16 isolates (22% of the selected isolates & 11% of the overall culture collection) were able to transfer resistance by conjugation, and these were distributed across all cohort groups. Resistance profiles of the transconjugants, determined by susceptibility testing on the transconjugant, were identical to those of the donors (Table 5).

4. Discussion

Previous studies have given rise to conflicting conclusions as to whether or not hospitalization is associated with an increase in antibiotic resistance in bacteria (Bruinsma et al., 2003; Koterba et al., 1986). Factors other than the use of antimicrobial drugs could influence the maintenance and development of antibiotic resistance of enteric bacteria in the gastrointestinal tract (Dewulf et al., 2007). The use of antibiotics in animals is of concern (Mora et al., 2005) since resistant organisms might be excreted in the faeces of animals, following administration of antimicrobials, and contribute to the reservoir of resistant bacteria in the environment (Ahmed et al., 2010). Resistant bacteria could be selected or acquired in the hospital environment and may subsequently be disseminated to the horses' home environments.

No.	Origin	Resistance phenotype	Donor genes (previously identified by PCR)
1,	NON-GI-	AMP	*Tem*
2	NON-GI-	AMP	*Tem*
3	NON-GI-	MDR	*dfr1,dfr12,tetA,tem,catI*
4	NON-GI-	MDR	*dfr1,dfr12,tetA,tem*
5	NON-GI-	MDR	*dfr1,dfr12,tetA,tem,catI*
6	NON-GI-	MDR	*tem*
7	NON-GI-	MDR	*dfr1,dfr12,tetA,tem*
8	GI+	AMP	*tem*
9	GI+	AMP,TRI	*dfr(7-17)*
10	GI+	AMP,TRI	*not identified*
11	GI+	AMP	*tem*
12	GI+	AMP	*tem*
13	GI+	AMP	*tem*
14	GI+	AMP,TRI	*dfr1*
15	GI+	AMP,TRI	*tem*
16	NON-GI +	AMP	*tem*

Table 5. Characteristics of isolates showing of transferable resistance.

This study and in contrast with others found no obvious association between antibiotic treatment, or clinical condition, and resistance profiles in faecal *E. coli*. This may be due to the relatively small sample size, or because horses entering the PLEH are largely referral cases and likely to have received antibiotic therapy prior to admission.

However, overall resistance to most individual antibiotics, and the proportion of MDR isolates increased during hospitalization and thereafter decreased during convalescence in the home environment. Recent studies by Dunowska et al, on horses, concluded that both hospitalization and antimicrobial administration were associated with the shedding of antimicrobial resistance *E. coli* strains of faecal origin (Dunowska et al., 2006). An earlier study, from a university equine hospital, found that the rate of resistance amongst *E. coli* and *Klebsiella* was higher at day seven of hospitalization compared to day one (Koterba et al., 1986). This may be due to selection during hospitalization through antibiotic therapy, and also the ready availability of resistant isolates in the hospital environment. It would be interesting to undertake PFGE analysis of the *E. coli* over time to investigate whether resistance is due to infection with resistant strains or horizontal transmission of resistance to the existing gut flora.

Antimicrobials select for resistance (Tenover et al., 2006) but the restriction of antimicrobials does not necessarily reduce antimicrobial resistance (Hoyle et al., 2006). In our study, the prevalence of resistant *E. coli* dropped markedly after discharge from the hospital, which may suggest that both the increase and decrease in resistance are due to turnover of *E. coli* between the gut and the environment.

E. coli with simple and multiple antimicrobial resistance (MDR) has been widely documented (Fofana et al., 2006; Ahmed et al., 2010). Bacteria can acquire or develop resistance to antimicrobials in different ways, including acquisition of resistant genes. *E. coli*

has been indicated as a possible reservoir for antimicrobial resistance genes and might play a role in the spreading of such determinants to other bacteria (Ahmed et al., 2010). The flora of healthy animals has also been implicated as a reservoir of antibiotic resistance genes (De Graef et al., 2004) and resistance transfer has been shown to occur between different animal species on farm premises (Hoyle et al., 2006). *E. coli* of animal origin with resistance to antibiotics and multiple antibiotics has been widely documented (Mora et al., 2005). The importance of farm animals in the spread of resistance to human populations is increased by worldwide reports of mobile genetic elements in animals raised for human consumption (Roest et al., 2007).

Our results for MICs and the genetic determination of resistance, suggest that, resistance was due to commonly reported genes causing such resistance in *E. coli* and other bacteria. It is interesting to note, that while some MDR transferred in the conjugation studies, many transconjugants were resistant to either ampicillin alone or ampicillin and trimethoprim (table 5). This suggests that both resistance profiles are encoded on mobile genetic elements. Horses in the GI+ and non-GI+ groups were the donors for most of the Amp and Amp/Trim transconjugants, and all the horses in both groups received therapy with cephalosporin drugs. It may, therefore be that these isolates represent either an endemic strain in the hospital, or an endemic plasmid moving rapidly between horses.

Multiple drug resistance phenotypes have been shown to be related to certain antibiotic drugs such as streptomycin and tetracycline (Mora et al., 2005). Also the resistance to a single antibiotic (i.e. tetracycline), in commensal *E. coli*, is linked to other antimicrobial resistances (e.g. ampicillin, trimethoprim and sulphonamides) (Dewulf et al., 2007). The *dfrA1, dfrA12, dfrA15* and *dfrA17* genes are documented to be carried on mobile genetic elements (i.e. integron classes), harboring resistance genes to at least three antimicrobials, and thus conferring multiple resistance (Ahmed et al., 2009). Other antibiotic resistances (i.e. ampicillin resistance) although found, were not strongly related to the presence of mobile genetic elements (Hoyle et al., 2005). Our PCR results in this study revealed similar observations within our collection of *E.coli* strains to other studies, although the conjugation results show that even isolates with single resistance (to ampicillin) transferred resistance (although not MDR). Thus, mobile genetic elements could also be responsible for single resistance and antimicrobial therapy might have resulted in such selection. The type of resistance and the identified genes (i.e. ampicillin resistance) could also be related to the type of antimicrobial therapy administered (e.g. cephalosporins). Such revelations, if proven by further studies, would mean that this kind of element may acquire further resistance genes in the future and help the dissemination and development of antibiotic resistance.

The importance of mobile gene pools in the spread of antibiotic resistance has been highlighted through comprehensive genomic analysis (Fricke et al., 2008). In our survey, a high proportion of the isolates tested in conjugation experiments were able to transfer resistance. *Dfr17* was the most prevalent trimethoprim resistance gene identified among the positive PCR isolates and *dfr1* was the second most prevalent. *tetA* was the most prevalent tetracycline resistant gene. This might indicate that the *dfr*17 and *tetA* resistant genes are more involved in the MDR mechanisms and most likely to be integrated within mobile genetic elements. The *tem* gene was also the most prevalent ampicillin resistant gene among the isolates and the *catI* gene was mostly found in MDR isolates. The *dfr17* is extensively reported to be involved in mobile genetic elements (Van et al., 2007). This, along with the

conjugation results, suggests that these elements are present in the hospital environment or that they are already constituents of the horses' intestinal flora. The referral hospital deals with horses in the area and horses are likely to be referred more than once to this hospital, which might lead to increases in the dissemination of resistance phenotypes in horses. The similarity between MDR phenotypes among collected strains can be epidemiologically important and molecular characterization (i.e. PFGE) in future studies will enhance our understanding of the phenomena.

The florfenicol resistant isolates were positive by PCR (five out of seven were positive for *catI*) and the mechanisms of this resistance require further investigation. However florfenicol resistance has been documented in *E. coli* of animal origin (Singer et al., 2004) and it has been shown that *floR* genetic determinants and others (i.e. *cmlA, cat1, cat2*) were also largely related to florfenicol resistance (Li et al., 2007). Others have shown that *floR* mediated resistance to chloramphenicol and florfenicol is plasmid mediated and also carries resistance to other genes (Blickwede et al., 2007; Kehrenberg et al., 2008). Recent molecular analyses have suggested that florfenicol resistance is strongly due to horizontal rather than clonal dissemination (Kehrenberg et al., 2008). This correlates with our results, in that florfenicol resistance is entirely documented among MDR isolates (although not proven transconjugants by our experiments). The horses in this study had never been treated with these classes of drugs, implicating a mobile genetic system in the acquisition of resistance from other animals or the environment.

5. Conclusions

No association between therapy and resistance profile was found in this study. However, the prevalence of antimicrobial resistance, and of MDR strains, did increase during hospitalization and subsequently decreased upon release from hospital. Thus therapy and the general environment of the hospital do appear to select for resistance and resistant isolates may disseminate once horses have been discharged, leading to clinical and public health concerns.

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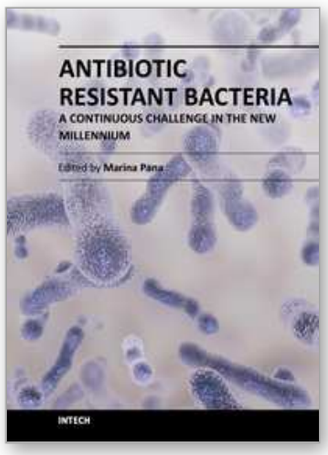

Antibiotic Resistant Bacteria - A Continuous Challenge in the New Millennium

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Antibiotic-resistant bacterial strains remain a major global threat, despite the prevention, diagnosis and antibiotherapy, which have improved considerably. In this thematic issue, the scientists present their results of accomplished studies, in order to provide an updated overview of scientific information and also, to exchange views on new strategies for interventions in antibiotic-resistant bacterial strains cases and outbreaks. As a consequence, the recently developed techniques in this field will contribute to a considerable progress in medical research.

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