

TRANSIENT INTESTINAL CARRIAGE AFTER INGESTION OF ANTIBIOTIC-RESISTANT *ENTEROCOCCUS FAECIUM* FROM CHICKEN AND PORK

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ABSTRACT

Background Antibiotic-resistant enterococci are often present in retail meats, but it is unclear whether the ingestion of these contaminants leads to sustained intestinal carriage.

Methods We conducted a randomized, double-blind study in 18 healthy volunteers. Six ingested a mixture of 10^7 colony-forming units (CFU) of two glycopeptide-resistant strains of *Enterococcus faecium* obtained from chicken purchased at a grocery store, six ingested 10^7 CFU of a streptogramin-resistant strain of *E. faecium* obtained from a pig at slaughter, and six ingested 10^7 CFU of a glycopeptide-susceptible and streptogramin-susceptible strain of *E. faecium* from chicken purchased at a grocery store. Suspensions of enterococci were prepared in 250 ml of whole milk and were well within the amounts deemed acceptable by Danish food regulations. Stool samples were collected before exposure, daily for 1 week after ingestion, and at 14 and 35 days. Resistant enterococci in stools were identified by selective culture techniques; further molecular characterization of the organisms was also conducted.

Results At the outset, none of the subjects were colonized with glycopeptide-resistant or streptogramin-resistant *E. faecium*. After ingestion of the study strains, these same strains were isolated from the stools of all subjects, in various concentrations. The test strain was isolated in stool from 8 of 12 subjects on day 6, and from 1 of 12 on day 14. All stool samples were negative at 35 days.

Conclusions The ingestion of resistant *E. faecium* of animal origin leads to detectable concentrations of the resistant strain in stools for up to 14 days after ingestion. The organisms survive gastric passage and multiply. (N Engl J Med 2001;345:1161-6.)

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THE proportion of carriers of glycopeptide-resistant enterococcus species in the general population varies widely from country to country. Values range from 0 percent to a few percent in Sweden, the Netherlands, and the United States¹⁻³; 11.8 percent in France⁴; and up to 28 percent in Belgium.⁵ The reasons for the wide variation among countries are not obvious but are probably related to differences in selection pressure. These differences may stem from the use of antimicrobial agents to treat diseases in humans or to promote growth or prevent infection in animals used for food, with the acquisition of these resistant strains by humans through the food chain.

Antibiotics have been used to promote the growth of food animals since the 1950s. Public health officials, including officials of the World Health Organization, have recommended the discontinuation of this practice, but veterinarians, farmers, and the veterinary-drug industry have argued against such action, saying there is insufficient evidence to indicate that this practice has any adverse effects in humans. There is a large reservoir of antimicrobial-resistant bacteria in food animals and on meat and poultry sold in grocery stores,⁶ but there is also a lack of information about what happens when humans ingest these resistant bacteria. The finding that strains of glycopeptide-resistant enterococci similar to those found in food animals have been identified in the stools of humans suggests a connection between the two, but it has not been accepted as proof. There are few studies of the rates of survival, carriage, or excretion of enterococci after ingestion. We therefore designed a study to determine the outcomes of the ingestion by healthy volunteers of glycopeptide-resistant and streptogramin-resistant enterococci that are found in meat.

METHODS**Ethics**

The protocol was approved by the scientific ethics committee for Copenhagen and Frederiksberg municipalities. The following ethical issues were carefully discussed during this process. *Enterococcus faecium* is part of the normal human flora, and neither *E. faecium* nor glycopeptide-resistant enterococci are very virulent, especially in immunocompetent persons. Most people ingest large amounts of this bacterium in various foods. The amount of bacteria proposed for use in the study was less than 10 percent of the amount that the Danish Food Agency permits as acceptable in foods. Furthermore, *E. faecium* is used as a probiotic in one brand of yogurt (Gaio, Arla Foods, Viby, Denmark) and as a supplement (Idoform tablets, Ferrosan, Søborg, Denmark); both are sold widely to consumers. If any subject were to become clinically infected or to have sustained intestinal colonization with one of the test strains, treatment would be possible, since the glycopeptide-resistant strains proposed for use are susceptible to penicillin, ampicillin, and gentamicin and the streptogramin-resistant strain is susceptible to vancomycin and ampicillin.

Study Subjects

The study was conducted in March and April 2000. Eighteen healthy volunteers (11 men and 7 women) were recruited among employees at Statens Serum Institut and the Danish Veterinary Laboratory in Copenhagen. All subjects received written and oral

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information about the study. No economic or other incentives were offered in exchange for their participation. All subjects were more than 18 years of age and had normal intestinal function with no known enteric disorders and no signs of loose stools. In addition, none had received antibiotics within one month before the study, none took antibiotics or other drugs during the study, and none had a history of an allergic reaction to penicillin. None of them were engaged in laboratory work, and all provided written informed consent.

The subjects were randomly divided into three groups of six. Each subject in group 1 ingested a mixture of two strains of glycopeptide-resistant *E. faecium* (Danish Veterinary Laboratory strains 9730129 and 9731048). Both strains were obtained from chicken purchased at a grocery store and carried transposon 1546 (Tn1546)-mediated *vanA* vancomycin resistance, with a minimal inhibitory concentration (MIC) of more than 256 μg of vancomycin per milliliter. Both strains were susceptible to penicillin, ampicillin, and gentamicin.

Each subject in group 2 ingested one streptogramin-resistant strain of *E. faecium* (Danish Veterinary Laboratory strain 9730735) with *vatD*-mediated resistance to virginiamycin (MIC, 32 μg of virginiamycin per milliliter). This strain had been obtained from a pig carcass immediately after slaughter and was susceptible to vancomycin and ampicillin. All three resistant strains had been shown by filter mating to be able to transfer the resistance genes (*vanA* and *vatD*). The locations of the genes have not yet been confirmed but are probably large plasmids.

Finally, each subject in group 3 ingested one strain of *E. faecium* (Danish Veterinary Laboratory strain 9830902) that was susceptible to both glycopeptides (MIC, 0.5 μg of vancomycin per milliliter) and streptogramins (MIC, 2 μg of virginiamycin per milliliter). This strain had been obtained from chicken purchased at a grocery store. This group served as the control group. At 35°C the in vitro growth curves of the four study strains were similar (data not shown).

Preparation of *E. faecium* Suspensions

The four strains were incubated overnight at 35°C on 5 percent blood agar (Statens Serum Institut). A suspension of each strain was prepared in 0.9 percent saline (Statens Serum Institut), and the concentration was adjusted to approximately 10^8 colony-forming units (CFU) per milliliter (range, 7×10^7 to 1.1×10^8) with use of a colorimeter (model 254, Sherwood Scientific, Cambridge, United Kingdom). The concentration was later confirmed by serial dilutions with colony counts. Immediately before it was given to the subjects, enough of the suspension was added to 250 ml of whole milk to yield a total count of 10^7 bacteria. This second suspension in milk was ingested by each of the subjects during a light meal of white bread, butter, jam, cheese, and Danish pastry. The suspensions were prepared by an independent investigator, and neither the subjects nor the investigators involved in the study knew the identity of the suspensions.

Collection and Culture of Stool Samples

Stool samples were collected within 48 hours before the subjects ingested the suspension, daily during the week after ingestion (days 0 through 6), and at 14 and 35 days. All stool samples were suspended in sufficient buffered saline (Statens Serum Institut) with a pH of 7.38 to yield a stool suspension of 0.2 g of stool per milliliter. Table 1 lists the mediums used for the stool samples. The stool samples were analyzed in a blinded fashion, and the code was not broken until all samples had been analyzed.

For the analysis of the stool samples obtained during the two days before the subjects ingested the bacterial samples, a 3-ml suspension of stool was transferred to a cryotube with 10 percent glycerin (Merck, Darmstadt, Germany) and stored at -40°C. A 1-ml stool suspension was plated on bile esculin azide (BE) agar (Difco, Detroit) containing 32 μg of vancomycin per milliliter (I-04094, Merck), and a 1-ml stool suspension was plated on BE agar containing 6 μg of virginiamycin per milliliter (B980122, Pfizer, Rixensart, Belgium).

TABLE 1. SELECTIVE MEDIUMS USED FOR THE VARIOUS STOOL SAMPLES.*

MEDIUM	DAYS OF COLLECTION OF STOOL SAMPLE
Bile esculin azide agar	0 to 6, 14
Bile esculin azide agar plus vancomycin (32 $\mu\text{g}/\text{ml}$)	-1, 35
Bile esculin azide agar plus vancomycin (32 $\mu\text{g}/\text{ml}$), virginiamycin (6 $\mu\text{g}/\text{ml}$), and erythromycin (32 $\mu\text{g}/\text{ml}$)	0 to 6, 14
Bile esculin azide agar plus virginiamycin (6 $\mu\text{g}/\text{ml}$)	-1, 35
Enterococcosel broth plus vancomycin (32 $\mu\text{g}/\text{ml}$), erythromycin (32 $\mu\text{g}/\text{ml}$), and aztreonam (60 $\mu\text{g}/\text{ml}$)	0 to 6, 14, 35
Enterococcosel plus virginiamycin (6 $\mu\text{g}/\text{ml}$), erythromycin (32 $\mu\text{g}/\text{ml}$), and aztreonam (60 $\mu\text{g}/\text{ml}$)	14, 35

*Results from stool samples obtained within 48 hours before the study meal are plotted as day -1. Results from stool samples obtained less than 24 hours after the study meal are plotted as day 0.

For the analysis of stool samples obtained on days 0 through 6 and day 14, a 1-ml stool suspension was transferred to a cryotube with 10 percent glycerin and stored at -40°C. A 500- μl stool suspension was transferred to 5 ml of Enterococcosel broth (Becton Dickinson, Oxford, United Kingdom) containing 32 μg of vancomycin per milliliter, 32 μg of erythromycin per milliliter (E-6376, Sigma, St. Louis), and 60 μg of aztreonam per milliliter (155960, Bristol-Myers Squibb, New York). A 100- μl stool suspension was plated on BE agar; a 250- μl suspension was plated on BE agar containing 32 μg of vancomycin per milliliter; a 250- μl suspension was plated on BE agar containing 32 μg of vancomycin per milliliter, 6 μg of virginiamycin per milliliter, and 32 μg of erythromycin per milliliter; and a 250- μl suspension was plated on BE agar containing 6 μg of virginiamycin per milliliter. In addition, a 500- μl stool suspension from day 14 was transferred to 5 ml of Enterococcosel broth containing 6 μg of virginiamycin per milliliter, 32 μg of erythromycin per milliliter, and 60 μg of aztreonam per milliliter.

For the analysis of the stool samples obtained on day 35, a 4-ml stool suspension was transferred to a cryotube with 10 percent glycerin and stored at -40°C. A 1-ml stool suspension was plated on BE agar containing 32 μg of vancomycin per milliliter and on BE agar containing 6 μg of virginiamycin per milliliter, and a 500- μl sample was transferred to both Enterococcosel broth containing 32 μg of vancomycin per milliliter, 32 μg of erythromycin per milliliter, and 60 μg of aztreonam per milliliter and Enterococcosel broth containing 6 μg of virginiamycin per milliliter, 32 μg of erythromycin per milliliter, and 60 μg of aztreonam per milliliter. The tubes of Enterococcosel broth and agar plates were incubated at 35°C for 48 hours.

If there was no bacterial growth on the agar plates, 100 μl of Enterococcosel broth (Enterococcosel broth containing 32 μg of vancomycin per milliliter, 32 μg of erythromycin per milliliter, and 60 μg of aztreonam per milliliter or Enterococcosel broth containing 6 μg of virginiamycin per milliliter, 32 μg of erythromycin per milliliter, and 60 μg of aztreonam per milliliter) was plated on BE agar and BE agar containing 32 μg of vancomycin per milliliter, 6 μg of virginiamycin per milliliter, and 32 μg of erythromycin per milliliter and incubated at 35°C for another 48 hours. The colonies were counted, and the bacteria were stored in 10 percent glycerin at -40°C.

The lower limit of detection of the resistant test strains was determined to be 40 CFU per gram of stool (4 bacteria in 0.1 g of feces showed growth in Enterococcosel broth containing 32 μg of vancomycin per milliliter, 32 μg of erythromycin per milliliter, and 60 μg of aztreonam per milliliter or Enterococcosel broth containing 6 μg of virginiamycin per milliliter, 32 μg of erythromycin per milliliter, and 60 μg of aztreonam per milliliter after 72 hours of incubation). Strains of *E. faecium* were identified according to routine laboratory procedures.

Typing of Strains from Stool Samples

Strains of *E. faecium* from BE agar containing 32 μg of vancomycin per milliliter; from BE agar containing 32 μg of vancomycin per milliliter, 6 μg of virginiamycin per milliliter, and 32 μg of erythromycin per milliliter; and from BE agar containing 6 μg of virginiamycin per milliliter were analyzed for *vanA* by the polymerase chain reaction according to the method of Poulsen et al.⁷ The enterococci were typed by pulsed-field gel electrophoresis. Chromosomal DNA was prepared and digested with *Sma*I. The sample plugs containing digested DNA were loaded on a 1 percent agarose gel and underwent electrophoresis at 200 V with a pulse wave of 760, an initial switching time of 5 seconds, a final switching time of 35 seconds, and a running time of approximately 30 hours. The gels were stained with ethidium bromide, and the DNA bands were identified with the use of ultraviolet light.

RESULTS

Changes over time in the concentrations of glycopeptide-resistant enterococci and streptogramin-resistant enterococci in the stool samples from the subjects are shown in Figures 1 and 2, respectively. The stool samples obtained before the ingestion of the bacterial suspensions were all obtained within 48 hours before the study meal and are plotted as day -1. Some of the subjects first defecated less than 24 hours after the study meal. Results from these stool samples are plotted as day 0. None of the subjects had any detectable adverse effect from the study.

One subject in group 2 (which was given the streptogramin-resistant strain) did not collect a preingestion stool sample but had no streptogramin-resistant strains of enterococci isolated in the next two stool samples. No glycopeptide-resistant or streptogramin-resistant enterococci were isolated from stool samples from any of the other 17 subjects before ingestion of the test strains.

All six subjects who were given the two strains of glycopeptide-resistant enterococci (group 1) had the test strains isolated from stool samples, in various concentrations, during days 0 to 6. The concentrations were maximal on day 2 and day 3, reaching 2×10^4 to 1×10^8 CFU per gram of stool. The concentrations on day 6 ranged from less than 40 CFU per gram of stool (the limit of detection) to 10^6 CFU per gram of stool. No vancomycin-resistant enterococci were isolated from this group on day 14 or day 35.

All six subjects who were given the streptogramin-resistant strain of enterococci (group 2) had the test strain isolated from stool samples during days 1 to 6. The concentrations were maximal on days 2, 3, 4, and 5, reaching approximately 10^4 CFU per gram of stool. In addition, the test strain was isolated from one subject on day 14 at a concentration of approximately 10^3 CFU per gram of stool. No streptogramin-resistant enterococci were isolated on day 35. All stool samples from this group were also tested for glycopeptide-resistant enterococci. One strain of glycopeptide-resistant enterococci was isolated at a concentration of 8×10^3 CFU per gram of stool on day 5 and day 6 in one subject. This strain had a different pattern on pulsed-field gel electrophoresis from the study strains of glycopeptide-resistant enterococci.

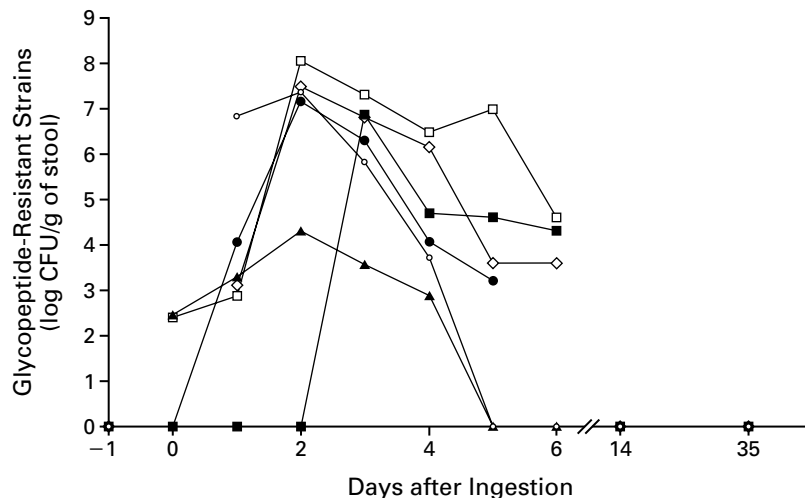


Figure 1. Fecal Excretion of a Mixture of Two Glycopeptide-Resistant Strains of *Enterococcus faecium* after Ingestion by Six Subjects.

Each curve shows the results for one subject. CFU denotes colony-forming units. Results from stool samples obtained within 48 hours before the study meal are plotted as day -1. Results from stool samples obtained less than 24 hours after the study meal are plotted as day 0. The solid squares with open centers represent the superposition of all six samples.

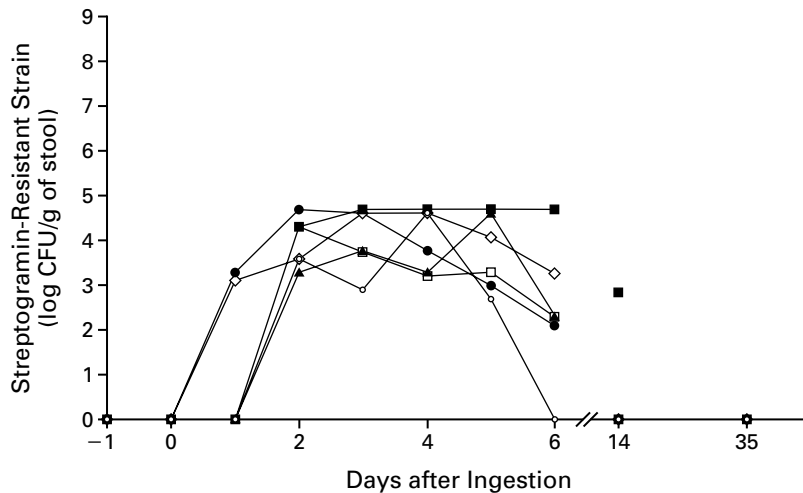


Figure 2. Fecal Excretion of a Streptogramin-Resistant Strain of *Enterococcus faecium* after Ingestion by Six Subjects.

Each curve shows the results for one subject. CFU denotes colony-forming units. Results from stool samples obtained within 48 hours before the study meal are plotted as day -1. Results from stool samples obtained less than 24 hours after the study meal are plotted as day 0. The solid squares with open centers represent the superposition of all six samples.

No glycopeptide-resistant or streptogramin-resistant strains of *E. faecium* were isolated from the stools of subjects in the control group, which was given one glycopeptide-susceptible and streptogramin-susceptible strain of *E. faecium*. The proportions of carriers in group 1 and group 2 were similar on a given study day.

A total of 147 isolates from group 1 and group 2 underwent pulsed-field gel electrophoresis to ensure that the resistant strains that were isolated were the ones that had been ingested. With the exception of the above-mentioned *vanA*-positive strain from one subject in group 2, all isolates had a pattern on pulsed-field gel electrophoresis that was identical to that of the study strains.

As a further control, 35 randomly selected enterococci isolated on two different days from all subjects in the control group were typed. Thirty of these had a pattern on pulsed-field gel electrophoresis that was indistinguishable from that of the control strain (data not shown). Since the control strain had no resistance marker, the concentration of the control strain could not be determined.

DISCUSSION

In this study of volunteers, we found that glycopeptide-resistant and streptogramin-resistant enterococci ingested with food in amounts similar to those present in meat sold in grocery stores can survive gastric passage, can multiply, and can be isolated in the feces for up to 14 days after ingestion. People com-

monly ingest glycopeptide-resistant enterococci and streptogramin-resistant enterococci as contaminants of meat. These microorganisms originate either from the animal itself or from contamination by human or environmental strains during processing. In Denmark in 1997, *E. faecium* was recovered from 50 percent of retail chicken samples, and 10 percent of these isolates were resistant to vancomycin. In the case of *E. faecium* isolated from chickens at slaughter, 48 percent of the isolates were resistant to vancomycin, and in random samples of retail chicken, 59 percent were resistant to virginiamycin.⁶

A number of studies demonstrate that enterococcal contamination of meat is widespread and that a large proportion of these enterococci are resistant to antimicrobial agents.⁷⁻¹³ However, the fate of these enterococci after ingestion is not certain. In a previous study, a single subject ingested 10^7 CFU of a glycopeptide-resistant strain of *E. faecium* of chicken origin; the strain was subsequently isolated from stool samples for three weeks.¹⁴ The microbiologic methods used in this study have been questioned, however.¹⁵

The study meal we gave to volunteers was meant to simulate a normal meal, with the study strains ingested as a suspension in milk. *E. faecium* can withstand adverse conditions, including low pH values in gastric contents. Although the relatively high content of fat in the study meal may have had a protective effect on the enterococci in lipid micelles, the composition of this meal was that of an average Danish breakfast.

The study strains were isolated from two different animal species — chickens and pigs. Furthermore, we included two different glycopeptide-resistant enterococci from poultry in order to preclude strain variation as a reason for the potential failure of colonization in the subjects. However, both strains of glycopeptide-resistant enterococci appeared to colonize equally well. Could these strains have been contaminants of human origin rather than animal origin? A prevalence study of carriage in the Danish population found only 1 carrier of glycopeptide-resistant enterococci among 287 healthy humans.⁶ Thus, contamination of human origin seems unlikely. In addition, the study strains from chicken had the base-pair variation in the *vanX* gene (part of the *vanA* gene complex) at position 8234 (G type) of the transposon Tn1546, which indicates an isolate of chicken origin.¹⁶ The other strain was obtained from a pig carcass immediately after slaughter, which makes contamination of human origin unlikely.

An inoculum of 10^7 bacteria was chosen for two reasons. First, a study by Berchieri used a similar inoculum and found that it was sufficient to colonize the human intestine for a limited period.¹⁴ Second, when suspended in 250 ml of whole milk, the concentration of enterococci (4×10^4 bacteria per milliliter) is similar to concentrations found in the chicken that consumers purchase at grocery stores.¹⁷

The study strains were isolated from the stools of every subject in the days after ingestion. In group 2, the maximal concentration of the study strain was two to three log lower than that in group 1. However, since in humans the average daily excretion of feces is approximately 150 g, the total excretion of the study strains during the first six days in subjects from both group 1 and group 2 exceeded the number of bacteria ingested in the case of 8 of 12 subjects. A major proportion of enterococci in feces from subjects in the control group also represented the study strain. The streptogramin-resistant strain tended to persist longer than the glycopeptide-resistant strains, and it would have been of interest to know the concentrations of the study strains from days 7 to 14. On day 14, only one subject, in group 2, remained positive for streptogramin-resistant enterococci, and all were negative by day 35. It remains possible that the study strains could persist in the gut of the subjects at concentrations below the limit of detection of 40 CFU per gram. If so, they might again be isolated if the subjects receive glycopeptide or streptogramin therapy in the future.

In one subject who received streptogramin-resistant enterococci, a strain of glycopeptide-resistant enterococci that differed from the study strains was isolated on day 5 and day 6. This subject worked at the Danish Veterinary Laboratory but was not directly engaged in laboratory work. The source and origin of the strain are unclear.

Transfer of resistance genes has been demonstrated

between strains of *E. faecium* in the gastrointestinal tract of gnotobiotic rats,¹⁸ among bacteroides species,¹⁹ between bacteroides species and gram-positive bacteria,¹⁹ and between *E. faecalis* and *Escherichia coli*,²⁰ and between *E. faecalis* and *Listeria monocytogenes* in gnotobiotic mice²¹; however, the risk of this event in humans is unknown. We did not find any indication of the transfer of resistance genes, since with one exception, the electrophoretic patterns of the strains isolated from the feces of the subjects were identical to those of the study strains, and the only glycopeptide-resistant enterococcus with a pattern that differed from those of the study strains was isolated from the feces of a subject who received a streptogramin-resistant strain but not a glycopeptide-resistant strain. However, the study was not designed to measure the rate of transfer of resistance genes, and it is impossible to rule out the occurrence of such a transfer during the study.

Our findings provide support for the recommendation that the use of antimicrobial agents for growth promotion in animals be discontinued. The only remaining step needed to show a direct effect of this practice on human health is to demonstrate the actual molecular transfer of the resistance determinant from the ingested bacteria to a human pathogen (i.e., a glycopeptide-resistant enterococcus) in a human.

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