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THE CONCEALING OF B-LACTAM ANTIBIOTICS AND PROGRESS OF DETECT METHODS

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ABSTRCT
The residue of β-lactam antibiotics has become an urgent issue of quality and safety of milk. β-lactamase conceal the existence of β-lactam antibiotics because it could decrease β-lactam antibiotics and change the antibiotics structure. As the development of antibiotic residues detection methods in milk, there is an increasing trend of adding β-lactamase into milk to decompose antibiotics. After the detection of β-lactamase become compulsory inspection items, the illegals began to consider how to conceal the existence of β-lactamase. Sulbactam could irreversibly bind with β-lactamase which could then decline free β-lactamase level even to zero. Therefore, after antibiotics and β-lactamase became mandatory testing items in milk, a phenomenon of adding sulbactam into β-lactamase-illegally-added milk to mask excessive antibiotics start to evolve. According to these situations, reviews were made on the concealing ofβ-lactam antibiotics and the corresponding detect method progress.

KEYWORDS: milk, β-lactam antibiotics, concealing, β-lactamase, sulbactam.

Milk has become indispensable food for its rich nutritional value in China. In recent years a series of milk safety problems emerged among which antibiotics residue was one of the most prominent. Antibiotics could effectively decrease cows' incidence of a disease andβ-lactam, represented by penicillin, with antimicrobial broad spectrum and cheap adantages, has been frequently over-dosage utilized and therefore has been the most common antibiotics residue.[1] β-lactam antibiotics would transmit and accumulate through feed-breed-process-transportation-storage food chain for its nodegradable property. Although antibiotics generally don't exert acute toxic effects on humans, frequently intake antibiotics-containing milk nearly equal to long-term usage of low-dosage antibiotics which may result accumulation in bodies. When the accumulation of antibiotics reaches certain level it would cause several acute and chronic poisoning in humans such as decreased immunity caused by antibiotic resistance of pathogenic bacteria in vivo, flora imbalance caused by disturbance the environment balance, anaphylactic reaction and hormone disorder allergy in susceptible populations.[2] Therefore, the maximum residue limits of 8 kindsβ-lactam antibiotics in milk were specificly regulated and antibiotics residue was mandatory testing project in Chinese law.

Currently detect methods in China related standards on β-lactam antibiotics such as liquid chromatography, mass spectrometry, microbiological method are all based on the spacial structure ofβ-lactam antibiotics and once its spacial structure altered, above mentioned methods become invalid.[3] There is obvious difference between antibiotic qualified milk and antibiotic excessive milk and many dairy companies adopt price cuts principle on antibiotic excessive milk. Under the interest driven, so called antibiotic disintegrant appear on the market to mask residual antibiotics so that make antibiotic excessive milk turn to antibiotic qualified milk. The component of above mentioned antibiotic disintegrant isβ-lactamase which is an ecto-enzyme secreted byβ-lactam antibiotics resistant strains. This ecto-enzyme could make conventional β-lactam detection methods invalid through destroying amide linkage of β-lactam and it still could selectively decreaseβ-lactam even after million times' dilution. Early in 1985, this enzyme was discovered to decrease residual penicillin in milk to undetectable level with conventional detection methods.[4] This is the 1st concealing means to mask excessive antibiotics.

There are two resouces of β-lactamase including endogenous one which is produced by related bacterium in milk and exogenous one which is artificial adding to mask antibiotics in milk by unscrupulous traders.[5] β-lactamase producing bacterium are mainly *Escherichia coli* and *Klebsiella pneumoniae* which havn't been detected in milk productes under currently manufacture process and preservation technology. In other words,

there is no background expression ofβ-lactamase in milk. Furthermore, it has been demonstrated that antibiotic destroyer found in milk was not endogenousβ-lactamase but artificial adding.[6] Since the report of β-lactamase'ability to decrease penicillin in milk, this enzyme is been intensively investigated: gene recombinant high efficient expression strain has been reconstructed and mature fermentation and purification technology has been established. Dozens of manufacture factories reached a certain scale utilizing above technology emerged in China and several of them even advertized selling residual antibiotics resist agent which is industry commonly known as antibiotic destroyer or golden magnolia enzyme.

Although there is no definite theory about thespecific hazard to humans of addingβ-lactamase in milk, its potential hazards lie in the below four aspects. The first one lies in that penicilloic acid, the decomposition products of β-lactamase is one of the main substances that cause human penicillin allergy.[7,8] The second one lie in that the adding of β-lactamase is highly possible of introducing microorganisms as this enzyme is synthesized by bacterium. The third one lies in lack of safety data for human body resulted insufficiency investigation on this enzyme. The fourth one lies in that the misapplication of β-lactamase encouraged misapplication of antibiotics in milk. Therefore, β-lactamase is not allowed to use in food by International Codex Alimentarius Commission, the European Union and the United States. β-lactamase is not included in the food enzyme list in Chinese Standards for Food Additives - GB2760. That is to say, β-lactamase is illegal food additives. Not only that, Chinese Ministry of Health (of that time) released list of potential illegal dietary supplements that contain inedible and/or unsafe ingredients (part I) and list of potential illegal dietary supplements that contain inedible and/or unsafe ingredients (part I) after melamine incident in 2008. β-lactamase are included in the list of part I. It can be seen from the above measures that the illegal use ofβ-lactamase has been a problem that can not be ignored.

Nowadays because of the national conditions and out of the public's strong concern about food safety and the grim situation of production, the detection of β-lactamase has become compulsory inspection item in milk. At present, frequently used β-lactamase detection methods include cylinder plate method (microbioassay), iodometric method, acidimetry method, high efficiency liquid chromatography and immunological method. The principle of cylinder plate method is based on the inhibition of *Micrococcus luteus* growth by penicillin and production of antibacterial circle and that sulbactam could specificly inhibite the activity ofβ-lactamase. Therefore it can be indirectly evaluated whether or not containβ-lactamase through comparing the antibacterial circle difference between penicillin samples adding sulbactam and penicillin samples without adding sulbactam. The principle of iodometric method is thatβ-lactamase enzymatic hydrolysates of penicillin, penicilloic acid combines with iodine and compete free iodine with starch which makes blue iodine starch complex colourless. Iodometric method is specified method of detecting β-lactamase in Chinese Pharmacopoeia. In cephalosporin coloration method, the color of cephalosporin changed as cephalosporin was unlinked byβ-lactamase which could be used as the judgement basis ofβ-lactamase occurrence and characterization ofβ-lactamase content. The principle of acidimetry is thatβ -lactamase decrese β-lactam antibiotics and the generated acidic products lower solution pH value which causes the indicator change color. Therefore, the content of β-lactamase could be detected through measuring pH value changes. The principle of high efficiency liquid chromatography (HPLC) is also that β -lactamase decrese β-lactam antibiotics. Quantitative penicillin was added into sample before detection. If there is β-lactamase in sample, the content of penicillin would decrease for the enzymolysis byβ-lactamase. Therefore, the change of quantitative penicillin could be used as criterion of the occurrence ofβ-lactamase. In immunological methods, β-lactamase as hapten is specifically combind with antibody to produce antigen-antibody complex. Then the result of biological test based on immulogical react could be used to justify the content of β-lactamase. Through synthetical consideration about above mentioned methods, Chinese National Standards specified cylinder plate method (microbioassay) as standard method to detect β-lactamase in milk-related products. This method is high sensitive, easy to operate and result determinate. Cui et al obtained the minimum detectability of this method was 4 U/m L.[9]

Sulbactam used in cylinder plate method (microbioassay, Chinese National Standards) is a kind of typical irreversible competitive enzyme inhibitor toβ-lactamase which inhibits β-lactamase produced by gram positive and negative bacteria (except for *Pseudomonas aeruginosa*) and the activity ofβ-lactamase could not recover even after the deletion of sulbactam. However, if sulbactam was added into β-lactamase-containing-milk (β-lactamase was used to conceal excessive antibiotics), it could irreversibly combine withβ-lactamase so as lead to the decrease of free β-lactamase in solution even to zero which affect the detection of antibiotics residue. In this sense, it is highly possible for sulbactam to be used by illegals to mask antibiotics decomposer in milk, furthermore to mask excessive antibiotics in milk. Actually from spring 2009, it was found thatβ-lactamase inhibitor was added into raw milk.[10]

As the widely use of antibiotics, bacteriam developed protectiveβ-lactamase to inactive β-lactam antibiotics before their reach to action site, which is also the main mechanism of pathological bacteria tolerance toβ-lactam antibiotics. Afterwards, it was found that β-lactamase inhibitors could combine with β-lactamase produced by bacteria and then inactive the later which could

effectively increase the curative effect of β-lactam antibiotics. The structure of β-lactamase inhibitor is similar to that of β-lactam antibiotics.β-lactamase inhibitor possess antimicrobial activity and could strongly inhibit β-lactamase. At present, several β-lactam derivatives were reported asβ-lactamase inhibitors, however, only clavulanic acid, sulbactam and tazobactam have clinical effects and among these, sulbactam is the most widely used. The structure of sulbactam is composed of by aβ-lactam ring linked to a five-membered thiazole ring on which sulfur atom is oxidized to sulfone. Sulbactam belongs to nontypical β-lactam antibiotics with molecular weight of 255.22. Sulbactam has been applied clinically with its alone application to peripheral infection caused by *Neisseria gonorrhoeae* and *Neisseria meningitidis*. Its combined application with ampicillin or other β-lactam antibiotics protects antibiotics from destroying and thereafter improve antibacterial activity through inhibiting β-lactamase activity which could be used to solve the problem of drug resistance of pathogenic bacteria. For example, sulbactam could be used to cure infection caused by *Staphylococcus aureus*, *Bacteroides fragilis*, *Streptococcus pneumoniae* and *Proteus vulgaris* which are all resistant to ampicillin. Another example is the 3rd generation compound preparation of Cephalosporin cefoperazone Cefperazone-Sulbactam.

Nevertheless, the useness of sulbactam in milk-related products has not been aproved in China. and the useness of sulbactam in milk is illegal. Although there is no reports about the effects of adding sulbactam in milk on humans, from the clinical data it could cause elevation of transaminase, alkaline phosphatase, lactate dehydrogenase and could cause anaphylactic reaction such as skin rashes, drug fever. Thereafter, adding sulbactam in milk not only makes illegally addedβ-lactamase aimed to mask excessive antibiotics nondectable which lay dairy safety hidden, but also threat consumers' health directly. Moreover, relative to the use of β-lactamase nearly implicit rule, the use of sulbactam is under a more secluded state. Just because of this, the danger of adding sulbactam in milk has not attracted enough attention, however, it has become the 2nd concealing means to mask excessive antibiotics.

In order to secure milk safety and to ensure consumers' health, timely detection and extensive supervision are effective ways to prevent and control the harmness of excessive antibiotics and thus it is urgent to establish detection methods of sulbactam in milk. From current literatures, the detection methods of sulbactam in milk include high efficiency liquid chromatography (HPLC).[10-12] liquid chromatography mass spectrometry.[13-15] microbioassay.[16] etc. Among these methods, HPLC and liquid chromatography mass spectrometry could quantify the content of sulbactam accurately and quickly, but sample pretreatment is complex and the equipment is expensive so it is time-consuming and high cost to complete an analysis. In traditional microbioassay, samples to be tested are added withβ-lactamase and penicillin G and control samples without β-lactamase and penicillin G. The content of sulbactam could be detected by comparing the inhibition zone diameter difference between samples to be tested and control samples. The advantages of this method is simple sample processing and easy to operate but it is timeconsuming and lack of specifity.

Hence it can be seen that as for the illega adding β-lactamase to mask excessive antibiotics in milk become plain in the marcket, the content ofβ-lactamase became the mandatory item, illegals try to add β-lactamase inhibitor sulbactam with ultimate aim to conceal excessive β-lactam antibiotics. In this sense, as for ensuring milk quality and consumers' health, timely detect and extensive supervision are effective ways to prevent and control the harmness of excessive antibiotics in milk.

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