SN

中华人民共和国出入境检验检疫行业标准

SN/T 0865—2000

进出口食品中肉毒梭菌及其肉毒毒素的检验方法

Method for the determination of *Clostridium botulinum* and botulinum toxin in foods for import and export

2000-06-22 发布　　2000-11-01 实施

中华人民共和国国家出入境检验检疫局　发布

前　　言

本标准是根据GB/T 1.1—1993《标准化工作导则　第1单元:标准的起草与表述规则　第1部分:标准编写的基本规定》对原专业标准ZB X09 005—1986《出口食品肉毒梭菌及其毒素检验方法》进行修订的。

本标准从实施之日起,同时代替ZB X09 005—1986。

本标准的附录A是标准的附录。

本标准由中华人民共和国出入境检验检疫局提出并归口。

本标准起草单位:中华人民共和国湖南出入境检验检疫局。

本标准主要起草人:朱金国、欧阳健、杨建功。

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1 范围

本标准规定了进出口食品中肉毒梭菌及其肉毒毒素的检验方法。

本标准适合于各种进出口食品及其原料中的肉毒梭菌和肉毒毒素的检验，有专门规定的检验方法除外。

2 引用标准

下列标准所包含的条文，通过在本标准中引用而构成为本标准的条文。本标准出版时，所示版本均为有效。所有标准都会被修订，使用本标准的各方应探讨使用下列标准最新版本的可能性。

GB 4789.26—1994 食品卫生微生物学检验 罐头食品商业无菌的检验

SN 0330—1994 出口食品中微生物学检验通则

公职分析化学家协会(AOAC)官方分析方法(1995) 食品中的肉毒梭菌及其毒素(微生物学方法)[AOAC Official Methods of Analysis(1995) 977.26;Clostridium Botulinum and Its Toxins in Foods (Microbiological Method)]

3 定义

本标准采用下列定义。

3.1 肉毒梭菌 clostridium botulinum

一种专性厌氧生长并产生芽胞的革兰氏阳性杆菌，属厌氧性梭状芽胞杆菌属，在适宜的培养基及特定的环境条件下产生肉毒毒素。

3.2 肉毒毒素 botulinum toxin

由肉毒梭菌产生的多类型的高分子不耐热蛋白质，为一类对人类、高等哺乳动物和鱼类都具有很强毒性的神经麻痹毒素。

4 样品准备和制备

4.1 初步检查

除未打开的罐装食品外，样品要冷藏直到检验。对未打开的罐装食品，有严重膨胀和有爆裂危险的必须冷藏。检验前应记录产品名称、生产厂名、样品来源、产品的生产批号和代号以及容器的情况。对容器进行清洁并作上供鉴别的标志。

4.2 固体食品

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用无菌操作方法将固体食品样品移入灭菌研钵中，加入等量的明胶磷酸盐缓冲液，并用灭菌研杵研磨，以备接种。亦可用灭菌镊子取小块的食品直接放入增菌肉汤。

4.3 液体食品

用灭菌吸管直接将液体食品接种到培养基中。

4.4 罐装食品

剥去罐头上的标签，检查外部缺陷，并作记录描述。用肥皂粉（或去污消毒液）和水清洗罐头，并用消毒液（有效氯浓度为 100 mg/L 的次氯酸钠溶液）擦工作台面。将洗净并擦干的罐头放在工作台上，同时进行编号标示。

用碘酒或其他有效的消毒剂先在罐头无代号的一端进行消毒，几分钟后再除去消毒剂。然后将罐头的这一端放在火焰上加热，直至上面的凝结水完全蒸发掉。若罐头已经膨胀和变形，打开前应进行适当冷却。操作时使其垂直侧壁焊缝背向操作人员，用火焰烧时应特别小心，以避免罐头爆裂。用 70%酒精浸湿的棉球擦拭开罐器手柄和刀刃，并用火焰充分烧灼金属部分。用开罐器在罐头经消毒加热处理的部位开一个大小适宜的孔（不得损伤罐盖卷边）。打开胖罐时，可在开罐处加盖清洁灭菌的纱布，以防内容物外溅。不移动罐头，立即用无菌操作取出食品放入培养基中。

4.5 样品的外观和气味检查

检查是否有任何腐败现象，但不得品尝样品。记录检查结果。

4.6 保存样品

接种样品后，以无菌操作取至少 25 g 样品放入灭菌样品瓶，置于－18℃下冷冻保存，以备后用。

5 检验方法

5.1 原理

当肉毒毒素与相应的抗毒素混合后，发生特异性结合，致使毒素的毒性全被抗毒素中和而失去毒力。以含有大于 1 个小白鼠最小致死量（MLD）的肉毒毒素的食品或培养物的提取液，注射于小白鼠腹腔内，在出现肉毒中毒症状之后，于 96 h 内死亡。相应的抗毒素能中和肉毒毒素并能保护小白鼠免于出现症状，而其他抗毒素则不能。食品中存活的芽胞能在厌氧的环境和适宜的培养条件下生长并产生毒素，得以检出和定型。

5.2 培养基和试剂

除特殊规定外，所有化学试剂均为分析纯，水为蒸馏水。

5.2.1 碘酒（碘 4%，溶于 70%乙醇中）。

5.2.2 庖肉培养基。

5.2.3 含有胰蛋白酶的胰酪蛋白胨葡萄糖酵母浸膏肉汤（TPGYT）。

5.2.4 厌氧卵黄琼脂。

5.2.5 明胶磷酸盐缓冲液，pH6.2。

5.2.6 无水乙醇。

5.2.7 革兰氏染色液。

5.2.8 结晶紫染色液。

5.2.9 美蓝染色液。

5.2.10 生理盐水。

5.2.11 多价肉毒毒素抗毒素（抗 A、B、C、D、E、F），可由卫生部兰州生物制品研究所和美国亚特兰大疾病控制中心获得。

5.2.12 胰蛋白酶溶液。

5.2.13 1 mol/L 氢氧化钠溶液。

5.2.14 1 mol/L 盐酸。

5.3 设备和材料

5.3.1 细菌学开罐器。

5.3.2 研钵和研杵。

5.3.3 吸管:1.0,5.0,10.0 和 25.0 mL。

5.3.4 培养试管(应有一些是带螺旋帽的)。

5.3.5 厌氧培养装置。

5.3.6 恒温培养箱,(26±1)℃及(35±1)℃。

5.3.7 显微镜(相差或明视野)。

5.3.8 平皿,皿底直径为 90 mm 或 100 mm。

5.3.9 高速冷冻离心机。

5.3.10 用于接种小白鼠的注射器,1.0 mL 或 3.0 mL,带有 5 号针头。

5.3.11 小白鼠,体重约 15～20 g(每一试验批应使用同一品系和同一性别的小白鼠)。

5.4 检验步骤

5.4.1 肉毒梭菌的检出

5.4.1.1 增菌培养

接种前,先将增菌培养基煮沸 10 min～15 min,以排除溶解于培养基中的氧,并迅速冷却,切勿摇动。每 15 mL 增菌肉汤中接种 1～2 g 固体食品或 1～2 mL 液体食品,接种时将接种物慢慢接入肉汤液面之下,每份样品接种两管庖肉培养基,置(35±1)℃培养,再按同样方法接种两管 TPGYT 肉汤,置(26±1)℃培养。

5.4.1.2 培养物的检查

培养 5 天后,检查培养物的浊度、产气、肉粒的消化并注意产生的气味。用显微镜检查经革兰氏、结晶紫或美蓝染色的培养物涂片,或取培养物以湿片在高倍相差显微镜下,观察菌体形态,并注意是否有典型的梭状菌、是否形成芽胞和芽胞形成的程度以及芽胞在菌体内的部位。同时对每一培养物作毒素检测。通常培养 5 天后是肉毒梭菌的活跃生长期,毒素的浓度最高,芽胞形成也达到高峰。为了分离纯培养物,应保留芽胞形成高峰期的增菌培养物并冷藏。如果培养 5 天的增菌液中没有细菌生长,应再培养 10 天以检出可能迟缓出芽的肉毒梭菌芽胞。

5.4.2 分离纯培养物

5.4.2.1 前处理

取(1～2) mL 培养液或原样品置于灭菌螺旋帽试管中,加入等量过滤除菌的无水乙醇。混匀,在室温下放置 1 h。也可取(1～2) mL 增菌培养物或原样品加热(80℃10 min～15 min)以破坏其繁殖体。但对非蛋白分解型肉毒梭菌不能加热处理。

5.4.2.2 涂平板

用接种环取 1～2 环经乙醇或加热处理的培养物或原样品在厌氧卵黄琼脂上划线接种,置厌氧条件下(35±1)℃培养 48 h。为了得到要挑取的单个菌落,必要时可将培养物稀释,为防止菌落蔓延成片,将琼脂平板表面干燥。

5.4.2.3 典型肉毒梭菌菌落的挑选

在每个平皿上挑取约 10 个单个的典型菌落。肉毒梭菌的菌落为隆起或扁平,光滑或粗糙。一般来说,它们容易蔓延生长并有不规则边缘。在卵黄培养基上用斜射光检查时,菌落表面通常呈虹彩样。亦称为珠色层。彩带通常向外延伸,继而,菌落产生不规则外形。除了珠色层外,在 C、D 和 E 型肉毒梭菌菌落周围通常有一个宽度为 2 mm～4 mm 的黄色沉淀晕。A 和 B 型菌菌落的沉淀晕一般较窄。由于梭状芽胞杆菌属的一些其他细菌虽不产生毒素,但能形成与肉毒梭菌的形态特征相似的菌落,挑选产毒菌落比较困难。

5.4.2.4 菌落接种

用灭菌接种环将挑选的每个菌落分别接种 TPGYT 肉汤和庖肉培养基各一管。按 5.4.1.1、5.4.1.2 所述的方法，将已接种的试管进行培养并作检查，然后按 5.4.3 中所述程序测试肉毒毒素。

5.4.2.5 确证试验

取 5.4.2.4 的培养物划线涂布于两个卵黄琼脂培养基，一个平板在(35±1)℃作厌氧培养，另一个平板则作(35±1)℃需氧培养。如果仅在厌氧培养的平板上有典型的肉毒梭菌菌落生长，而在有氧培养的平板上没有菌落生长，则培养物可能是纯的。在挑选的菌落中如果分离不出肉毒梭菌，意味着在增菌培养基里的混合菌相中，肉毒梭菌的数量相当少。再通过增菌，反复转种，肉毒梭菌的数量可能会增加到足以使该菌分离出来。纯培养物应以芽胞状态用无菌、干燥的石英海砂或玻璃珠吸附后冷藏、冷冻或冻干。

5.4.3 肉毒毒素的测定

5.4.3.1 样品制备

含有悬浮物的液态样品应当冷冻离心，取其上清液作毒素测定。固体食品要加等体积 pH6.2 明胶磷酸盐缓冲液，用预冷的研钵和研杵研磨，3 000 r/min，10 min～20 min 冷冻离心研磨的样品，用其上清液作毒素测定。

5.4.3.2 胰酶处理

测毒素前，用胰蛋白酶处理一部分食物上清液、液体食品或庖肉培养物。用 1 mol/L 氧氢化钠或 1 mol/L盐酸调节 pH 到 6.2。取每种待检上清液 1.8 mL 加 0.2 mL 饱和胰酶水溶液，于 37℃下孵育 1 h，间或轻轻摇动(饱和胰酶液制备：取 1 g 1∶250 胰酶放入到一个洁净的试管中，加 10 mL 蒸馏水，不时摇动，直到尽可能多的胰酶被溶解为止)。

对 TPGYT 培养物则不用胰蛋白酶处理，因为这种培养基已含有胰酶，进一步处理会降解培养物中已经充分活化的毒素。

5.4.3.3 测定

把一部分未处理的样品液或培养物分别用明胶磷酸盐缓冲液作 1∶2、1∶10 和 1∶100 稀释。把每份经胰酶处理的样品液或培养物也作同样的稀释。用 1.0 mL 或 3.0 mL 带有 5 号针头的注射器，取上述未稀释的液体和已稀释的不同浓度的液体各 0.5 mL 分别给两只小白鼠作腹腔内注射。取 1.5 mL 未经处理的样品上清液或培养物在 100℃加热 10 min。冷却后，取 0.5 mL 这种液体注射两只小白鼠。这两只小白鼠不应死亡，因为即便注射液中有肉毒毒素，经过加热处理已被灭活。

定时观察所有小白鼠 96 h，检查是否有肉毒中毒症状，记录症状和死亡情况。小白鼠肉毒中毒的典型症状通常在 24 h 内出现，典型症状是：毛发竖立、呼吸困难、四肢瘫痪；继而呼吸呈风箱式、腰部凹陷，宛若蜂腰；最终死于呼吸麻痹。小白鼠如没有肉毒中毒的临床症状而死亡，不能足以证明接种材料中含有肉毒毒素，有时，死亡是由于接种液中存在其他化学物质或由于外伤所致。如出现小白鼠猝死，以致症状不明显，或经 96 h 的观察后，如果除那些注射了热处理的材料外的所有小白鼠均死亡，那么就要用更高稀释度的上清液或培养物重复试验。

5.4.3.4 确证试验

采用小白鼠体内中和保护试验法进行可疑毒素样品确证实验。

不论是样品液或培养物，凡能致小白鼠发病、死亡者，取样进行适当稀释(检样的稀释应参考所用多价肉毒抗毒素的效价)。如果是测定经胰酶处理的样品，则需制备新鲜的经胰酶处理的被试液，因胰酶的持续作用可能破坏毒素。

在给小白鼠注射可疑毒素稀释液以前 30 min～60 min，分别取多价肉毒抗毒素 0.5 mL 给每只小白鼠作腹腔注射。

将各稀释度的可疑毒素样品液给注射了多价肉毒抗毒素的小白鼠作腹腔注射，每只小白鼠注射 0.5 mL，每个稀释度注射两只小白鼠。同时用每一稀释度的样品液注射两只未注射抗毒素的小白鼠作对照。

观察小白鼠 96 h，注意注射肉毒抗毒素小白鼠和对照小白鼠的中毒症状，并记录死亡情况。

注：如需对肉毒毒素作进一步的分型测定，可参照其它相关的标准方法进行测定。

5.5 结果解释

实验室检验旨在鉴定食品中的肉毒毒素和(或)菌体。

对肉毒梭菌的检出和鉴定必须以产毒试验的结果为依据。只有用肉毒毒素抗毒素保护的小白鼠免于肉毒中毒死亡，方能证实样品中有肉毒毒素存在。

如果多价肉毒抗毒素不能保护小白鼠，小白鼠可能是死于别的原因。如果经热处理和未经热处理的被试液都能使小白鼠死亡，可能是被试液中存在其他耐热毒素物质。但要特别注意耐热毒素物质掩盖肉毒毒素存在的可能性。

附 录 A
（标准的附录）
培养基和试剂的配制

A1 庖肉培养基

新鲜牛肉	500.0 g；
蛋白胨	30.0 g；
酵母浸膏	5.0 g；
磷酸二氢钠($NaH_2PO_4 \cdot H_2O$)	5.0 g；
葡萄糖	3.0 g；
可溶性淀粉	2.0 g；
蒸馏水	1 000.0 mL。

将新鲜除脂肪和筋膜的牛肉 500 g 切碎，加入蒸馏水。加热至沸点，再以文火煮 1 h。充分冷却，经纱布过滤，挤出余液。加入其他成分，用蒸馏水将液体体积补足至 1 000 mL。调节 pH 至 7.4，经粗滤纸过滤。可将肉汤和碎肉渣分别贮藏于冰箱内备用。在 15 mm×150 mm 试管中先加入碎肉渣至约 3 cm 高，然后加入肉汤，超过肉渣表面约 4 cm，上面覆盖一层液体石蜡，厚度为 0.3 cm～0.4 cm。在 121℃高压灭菌 20 min。

A2 含有胰蛋白酶的胰蛋白胨葡萄糖酵母浸膏肉汤(TPGYT)

A2.1 基础液

胰酪胨(trypticase)	50.0 g；
蛋白胨	5.0 g；
酵母浸膏	20.0 g；
葡萄糖	4.0 g；
硫乙醇酸钠	1.0 g；
蒸馏水	1 000.0 mL。

将固体成分溶于 1 000 mL 蒸馏水中，再分装 15 mm×150 mm 试管，每管 15 mL。上面覆盖一层液体石蜡，厚度为 0.3 cm～0.4 cm。在 121℃下高压灭菌 10 min。最终 pH 为 7.2±0.1。放冰箱内保存，若两周内不用则弃掉。临用前，将基础液用蒸气或煮沸加热 10 min～15 min，以排除游离氧，迅速冷却，以无菌操作每 15 mL 肉汤加入 1.0 mL 胰酶液。

A2.2 胰酶液

胰酶(1∶250)	1.5 g；
蒸馏水	100.0 mL。

将胰酶溶解于蒸馏水中，用 0.45 μm 微孔滤膜滤器过滤除菌。

A3 厌氧卵黄琼脂

A3.1 琼脂基础

酵母浸膏	5.0 g；
胰胨	5.0 g；
胨胨	20.0 g；
氯化钠	5.0 g；

琼脂　　20.0 g；

蒸馏水　　1 000.0 mL。

在121℃下高压灭菌15 min。最终pH为7.0±0.2。

A3.2　卵黄乳状液

用硬刷洗刷2～3个鸡蛋，沥干。将鸡蛋放在0.1%氯化汞溶液里浸泡1 h，取出沥干，再用70%酒精浸泡30 min。取出鸡蛋，以无菌操作打开，弃去蛋白。用注射器取出蛋黄，放入灭菌容器，加等量灭菌生理盐水，充分混合，存于4℃备用。

A3.3　培养基制备

每500 mL琼脂基础液(48℃～50℃)加80 mL卵黄乳状液，充分混合，制成平板。在室温下放置2天，或35℃下放置24 h。剔除污染的平板，将无菌平板存于冰箱。

A4　革兰氏染色液

A4.1　Hucker氏草酸铵结晶紫液

a）甲液：

结晶紫(染料含量90%)　　2.0 g；

95%乙醇　　20.0 mL。

b）乙液：

草酸铵　　0.8 g；

蒸馏水　　80.0 mL。

将甲液、乙液混合。放置24 h，经粗滤纸过滤。

c）革兰氏碘液：

碘　　1.0 g；

碘化钾　　2.0 g；

蒸馏水　　300.0 mL。

将碘化钾置研钵中，加入碘，用研杵研磨5 s～10 s；加1 mL蒸馏水研磨；加5 mL蒸馏水研磨，然后加10 mL蒸馏水再研磨。将此溶液装入试剂瓶。用蒸馏水淋洗研钵和研杵，并收集洗液，使溶液的总体积成为300 mL。

A4.2　Hucker氏对比染色液(母液)

沙黄　　2.5 g；

95%乙醇　　100.0 mL。

将10 mL母液加于90 mL蒸馏水中即成。

A5　结晶紫染色液

A5.1　结晶紫稀乙醇液

结晶紫(染料含量90%)　　2.0 g；

95%乙醇　　20.0 mL；

蒸馏水　　80.0 mL。

A5.2　草酸铵结晶紫(Hucker氏)液(见A4.1)

以上两者都被认为是稳定的作形态学检查的染色液。

A6　美蓝染色液(Loeffler氏)

a）甲液：

美蓝(染料含量90%)　　0.3 g；

95%乙醇　　30.0 mL。

b）乙液：

稀释的氢氧化钾(0.01%)　　100.0 mL；

将甲液、乙液混合即成。

A7 消毒剂

A7.1 碘酊

碘化钾　　10.0 g；

碘　　10.0 g；

70%乙醇　　500.0 mL。

A7.2 次氯酸钠溶液

次氯酸钠　　5.0 g～5.25 g；

蒸馏水　　100.0 mL。

A8 明胶磷酸盐缓冲液

明胶　　2.0 g；

磷酸氢二钠(Na_2HPO_4)　　4.0 g；

蒸馏水　　1 000.0 mL。

将明胶和磷酸盐加于蒸馏水中，稍加热使溶解。121℃高压灭菌 20 min。最终 pH 为 6.2。

A9 生理盐水

氯化钠　　8.5 g；

蒸馏水　　1 000.0 mL。

将氯化钠溶解于蒸馏水中。在 121℃高压灭菌 15 min，冷却至室温。

A10 1 mol/L 氢氧化钠溶液

氢氧化钠　　40.0 g；

溶解于蒸馏水，并加至 1 000 mL。用于调节培养基的 pH。

A11 1 mol/L 盐酸

盐酸(浓)　　89.0 mL；

加蒸馏水至 1 000 mL。

PREFACE

This standard is a revision of ZB X09 005—1986 "Method of the determination of *Clostridium botulinum* and botulinum toxin in food for export" in accordance with GB/T 1.1—1993 "Directives for the Work of Standardization—Unit 1:Drafting and Presentation of Standards—Part 1:General Rules for Drafting Standards".

This standard is intended to replace ZB X09 005—1986,starting from the date it is put into effect.

Annex A of this standard is an Appendix to the standard.

This standard was proposed and filed by the State Administration of Entry-Exit Inspection and Quarantine of the People's Republic of China.

This standard is drafted by Hunan Entry-Exit Inspection and Quarantine Bureau of the People's Republic of China.

Main draftsmen of this standard:Zhu Jinguo,Ou Yang jian,Yang Jiangong.

Professional Standard of Entry-Exit Inspection and Quarantine of the People's Republic of China

SN/T 0865—2000
In replacement of
ZB X09 005—1986

Method for the determination of *Clostridium botulinum* and botulinum toxin in foods for import and export

1 Scope

This standard lays down the method of detecting Clostridium botulinum and botulinum toxin in foods for export.

This standard applies to the inspection of Clostridium botulinum and botulinum toxin in all kinds of food for export and the material for making them, except those of which there is specified method for the inspection.

2 Standards Incorporated

Clauses in standards below-mentioned, having been incorporated in this standard, become its component parts. Editions of the cited standards will still be effective when this standard is published. As all standards are subjected to revisions, all parties concerned should study the possibilities of using the latest editions of the following standards:

GB 4879.26—1994 Microbiological examination of food hygiene—Examination of commercial sterilization of canned food

SN 0330—1994 General guidance for microbiological examination of export foods

AOAC official methods of analysis (1995) 977.26: Clostridium Botulinum and Its Toxins in Foods (Microbiological Method)

3 Definition

This standard adopts the following definitions:

3.1 Clostridium botulinum

A kind of strictly anaerobic spore-generating Gram-positive bacillus which produces botulinum toxin in suitable culture media under certain circumstances. It falls into the family of anaerobic clostridial bacilli.

3.2 Botulinum Toxin

Polytypic hypermolicular heat-labile protein produced by Clostridium botulinum. A kind of nerve paralyzing toxin, highly poisonous to human beings, advanced mammals and fish.

4 Preparation of sample

4.1 Preliminary Examination

Approved by the State Administration of Entry-Exit Inspection and Quarantine of the People's Republic of China, 06-22-2000

Implemented on 11-01-2000

Except for unopened canned foods, samples must be kept refrigerated until being examined. Unopened canned foods, unless badly swollen or in danger of bursting, need not be refrigerated. Before inspection, notes such as the product description, the name of the manufacturer, the source of the sample, the kind, size and condition of the container, the label, batch number, bale number or product code should be made. Containers should be rinsed and marks for identification made.

4.2 Solid foods

Aseptically transfer a portion of the sample solid food to a sterile mortar, add equal amount of gel-phosphate buffer and grind with a sterile pestle for inoculation. Adding small pieces of sample with sterile forceps directly into an enrichment broth is also acceptable.

4.3 Liquid foods

Inoculate the food with sterile pipets directly into culture media.

4.4 Canned foods

Remove label on the can, check for external faults and take necessary notes. Rinse the can with soap powder (or scouring disinfectant) and water. Put the clean and dried can on operation platform, which should be scoured with disinfectant solution (a NaClO solution with a valid chlorine concentration of 100 mg/L), number the can in the mean time.

Iodine solution or other effective disinfectants are applied to the non-coded end of the can for disinfection. After several minutes remove the disinfectants. Then put this end of the can on flame till complete evaporation of the surface moisture. If the can has swelled and deformed, cool properly before opening the can. Turn the vertical side seam away from the operator. To avoid bursting, special care must be taken when flaming the can. Disinfect the handle and blade of the specialized can-opener with cotton wool steeped in 70% alcohol and flame the metallic part thoroughly. Make a hole of appropriate size in the disinfected and heated part of the can with the opener (make sure not to wound the rolled rim of the cover). When opening a swollen can, cover the opening with sterile gauze to prevent spilling. Aseptically and immediately inoculate food from the can into the culture medium without moving the can.

4.5 Visual examination

Examine the appearance, odor for any evidence of decomposition. **DO NOT TASTE PRODUCT** under any circumstances. Make necessary notes.

4.6 Reserve sample

After inoculation, aseptically remove at least 25 g of the sample to the sterile sample jar, preserve it below minus 18℃ for further test, which may be needed later.

5 Detection Method

5.1 Principle

Botulinum toxin mixed with homologous antitoxin incurs idiotypic combinations, which result in full neutralization of the toxicity by the antitoxin. Mice injected intraperitoneally (IP) with food or extract containing ≥1 min. lethal dose (MLD) of botulinum toxin die 96 hr after exhibiting sequence of symptoms characteristic of botulinum intoxication. Homologous antitoxin will neutralize the botulinum toxin and protect mice from symptoms while other antitoxins will not. Viable spores in food will grow anaerobically and in suitable cultural circumstances and produce toxin, which is detected and typed.

5.2 Media and reagents

Unless specified, all chemical reagents are of analytical pure, and water is distilled.

5.2.1 Iodine (4% iodine dissolved in 70% alcohol).

5.2.2 Cooked meat broth.

5.2.3 Trypticase-peptone-glucose-yeast extract broth with trypsin(TPGYT).

5.2.4 Anaerobic egg yolk agar.

5.2.5 Gel-phosphate buffer, pH6.2.

5.2.6 Absolute alcohol.

5.2.7 Gram stain.

5.2.8 Crystal violet.

5.2.9 methylene blue.

5.2.10 physiological saline.

5.2.11 Polyvalent Clostridium botulinum antitoxin preparations (types A through F), available in Lanzhou Biological Product Research Center of the State Department of Health or in the Atlanta Disease Control Center, USA.

5.2.12 Trypsin solution.

5.2.13 1 mol/L NaOH solution.

5.2.14 1 mol/L HCl.

5.3 Apparatus and Materials.

5.3.1 bacteriological can opener.

5.3.2 mortar and pestle.

5.3.3 pipets in 1.0 mL, 5.0 mL, 10.0 mL and 25.0 mL.

5.3.4 culture test tubes (some should be with screw caps).

5.3.5 anaerobic jars.

5.3.6 incubators—(26±1)℃ and (35±1)℃.

5.3.7 microscopes (power phase contrast or bright field illumination).

5.3.8 Petri dishes of 90 mm or 100 mm in diameter.

5.3.9 high-speed, refrigerated centrifuge.

5.3.10 syringes of 1.0 mL or 3.0 mL, with 5 gage needles, for inoculating mice.

5.3.11 white mice of 15 g～20 g in weight. (Mice of the same breed and sex should be exclusively included in each experimental group).

5.4 Detection procedure

5.4.1 detection of viable Clostridium botulinum

5.4.1.1 Enrichment

Before inoculation, remove dissolved oxygen from media by steaming 10～15 minutes and cooling quickly without agitation. Inoculate per 15 mL of enrichment broth with 1～2 g solid food or 1～2 mL liquid food, introducing inoculum slowly beneath the surface of broth. Inoculate for each sample 2 tubes of cooked meat media and incubate at (35±1)℃. Similarly inoculate 2 tubes of HPGYT broth and incubate at (26±1)℃.

5.4.1.2 Examination

After 5 days, examine cultures for turbidity, gas production, digestion of meat particles, and odor. Also examine smear, stained by Gram stain, crystal violet or methylene blue, microscopically by wet mount under high power phase contrast or by bright field illumination. Observe morphology of organisms and note the existence of typical clostridial cells, the occurrence and relative extent of sporulation, and the location of spores within cells. Meanwhile determine toxins of each culture. Usually a 5-day incubation produces ushers in active growth and the highest concentration of toxin, as well as peak sporulation. Retain enrichment culture of the peak sporulation in refrigerator for pure culture isolation. If there is no growth after 5 days, incubate additional 10 days to detect possible delayed germination of C. botulinum spores.

5.4.2 Isolation of pure cultures

5.4.2.1 Pretreatment

Add equal volume of filter-sterilized absolute alcohol to 1～2 mL culture or sample in sterile screw-cap tube. Mix well and incubate at room temperature for 1 hour. Alternatively, heat 1～2 mL enrichment culture for 10～15 minutes at 80℃ to destroy vegetative cells. Do not use heat treatment for nonproteolytic type C. botulinum.

5.4.2.2 Plating

With inoculating loop, streak 1 or 2 loopfuls of alcohol/heat-treated culture or the original sample, diluted if necessary, to anaerobic egg yolk agar dried plates. Incubate the plates for 48 hours at (35±1)℃ under anaerobic condition. Dilute the culture, if necessary, to obtain isolated colonies for selection.

5.4.2.3 Selection of colonies

Select 10 isolated typical colonies from each petri dish. Typical C. botulinum colonies are raised or flat, smooth or rough, and commonly show some spreading and have irregular edge. On egg yolk media, colonies usually exhibit surface iridescence when examined by oblique light. This luster zone, referred to as "pearly layer", usually extends beyond and follows irregular contour of colony. Besides pearly zone, colonies of types C, D and E are ordinarily surrounded by wide (2～4 mm) zone of yellow pptn. Colonies of types A and B generally show smaller zone of yellow pptn. As some members of

genus Clostridium have typical morphological characteristics but do not produce toxins, it is rather difficult to select colonies that produce them.

5.4.2.4 Inoculation of colonies

With sterile transfer loop, inoculate each of the 10 selected colonies into a tube of sterile medium TPGYT broth, and a tube of cooked meat broth. Incubate and examine the inoculated tubes in accordance with the methods specified in 5.4.1.1 and 5.4.1.2, and detect the botulinum toxins according to the procedures laid down in 5.4.3.

5.4.2.5 Confirmation

Streak culture from 5.4.2.4 in 2 duplicate egg yolk agar plates, incubating 1 plate anaerobically and the other plate aerobically at (35±1)℃. If colonies typical of C. botulinum are found on anaerobic plate and no growth is found on aerobic plate, the culture may be pure. Failure to isolate C. botulinum from ≥1 of the selected colonies may indicate that its population relative to the enrichment medium of the mixed flora is low. Repeated serial transfers through additional enrichment steps may increase the number sufficiently to permit isolation. Suck spores in the pure culture with quartz sea sands, which are then stored either under refrigeration, or lyophilized.

5.4.3 Detection of Botulinum toxin

5.4.3.1 Preparation of sample

Centrifugalize under refrigeration liquid samples or extract containing suspended solids, use the supernate for toxin detection. Extract solid food with equal volume of pH6.2 gel-phoshate buffer, macerating with sterile, prechilled mortar and pestle. Centrifugalize under refrigeration the macerated sample at 3 000 r.p.m. for 10～20 minutes, and use the supernate for toxin detection.

5.4.3.2 Trypsin treatment

Before toxin detection, treat a part of the supernate, liquid food or cooked meat culture with trypsin. Adjust to pH 6.2 with 1 M NaOH or 1 M HCl. Mix 0.2 mL of saturated trypsin solution with 1.8 mL of each supernate and incubate at 37℃ for 1 hour, shaking gently once in a while. To prepare the saturated trypsin solution, disperse 1 g of trypsin (Difco 1 : 250) in 10 mL H_2O in a clean tube, shaking from time to time till no more trypsin could be dissolved.

Do not use trypsin treatment with TPGYT culture, which already contains trypsin. Further treatment may degrade any fully activated toxin present in culture.

5.4.3.3 Toxicity testing

Dilute portions of untreated sample solution or culture by 1 : 2, 1 : 10, 1 : 100 respectively, with gel-phosphate buffer. Dilute the trypsin-treated sample or culture in the same way. Inject intraperitoneally separate pairs of mice with the above-mentioned original and various diluted fluids, using 1.0 mL or 3.0 mL syringes with 5 gage needles. Heat 1.5 mL of the above-mentioned untreated sample supernate or culture for 10 minutes at 100℃. Cool, and inject a pair of mice each with 0.5 mL of the heated fluid. These mice should not die because botulinum toxin, if present, is inactivated by the heat treatment.

Observe the mice periodically for 96 hours, recording symptoms and time of deaths. Typical symptoms of botulism usually begin within 24 hours with ruffing or fur, followed in sequence by labored breathing, weakness and paralysis of limbs, wind-box breathing, collapse of waist like a wasp's, and final death due to respiratory failure. Death without symptoms of botulism is not sufficient evidence that the inoculated material contained botulinum toxin. Deaths may sometimes occur from other chemicals

present in the fluid or from trauma. If sudden deaths occur, leaving no detectable symptoms, or if after 96 hours all but the mice receiving the heated preparation have died, repeat toxicity test using supernate or culture of higher dilutions.

5.4.3.4 Confirmation test

Doubtful toxin samples can be ascertained by the internal neutralization protection test, carried out on mice.

Dilute portions of the sample fluid or culture, which bring about symptoms and deaths to the mice, proportionately to the value of the polyvalent C. botulinum. If the sample subjected to confirmation is trypsin-treated, it should be freshly prepared for the test because constant action of the trypsin may destroy the toxin.

Inject intraperitoneally each mouse with 0.5 mL of 1 of the polyvalent antitoxins, 30～60 minutes before challenging them with the injection of the dubious toxic dilutions.

Inject intraperitoneally pairs of mice protected by specific polyvalent antitoxin injection, with 0.5 mL of each of the dubious toxic sample dilutions. Mean while, inject pairs of unprotected mice with each toxic dilution as control.

Observe for 96 hours both the mice receiving antitoxin injections and the controls for symptoms of botulism and record manners of deaths.

Note: If further typing test is necessary, other relevant standards are ready references.

5.5 Interpretation

Lab detection is to examine food for the presence of C. botulinum and/or botulinum toxins.

The detection and determination of C. botulinum base on the result of toxin-producing test. The presence of C. botulinum toxins in sample food can be proved only when mice protected by polyvalent antitoxins do not die of botulism.

If polyvalent antitoxins do not protect mice, deaths may be due to some other cause. If both heated and unheated fluids cause death, some other toxic material in the tested fluids, which is not heat labile, could be responsible. But special attention should be paid to the possibility that heat stable toxic substance could mask botulinum toxin.

Appendix A
(Appendix to the Standard)
Media and Reagents

A1 Cooked meat broth

Fresh beef	500.0 g
Peptone	30.0 g
Yeast extract	5.0 g
$NaH_2PO_4 \cdot H_2O$	5.0 g
Glucose	3.0 g
Solvable starch	2.0 g
H_2O	1 000.0 mL

Use 500g of chopped fresh beef without fat, sinews or membrane, add H_2O, heat to boilling point, and continue heating with slow flame for 1 hour. Cool, filter through cheesecloth, pressing out excess liquid. Add other ingredients, dilute to 1 000 mL with H_2O. Adjust to pH 7.4. Filter through coarse paper. If desired, broth and meat dregs may be stored separately in freezer for future use. To 15 mm ×150 mm test tubes, add meat dregs to a height of 3 cm, and broth about 4 cm above the dregs surface, cover with a 0.3 cm～0.4 cm liquid paraffin layer. Autoclave at 121℃ for 20 minutes.

A2 Trypticase-peptone-glucose-yeast extract broth with trypsin(TPGYT)

Base fluid:

Trypticase	50.0 g
Peptone	5.0 g
Yeast extract	20.0 g
Glucose	4.0 g
Na thioglycollate	1.0 g
H_2O	1 000.0 mL

Dissolve the solid components into 1 000 mL H_2O and dispense 15 mL portions into 15 mm×150 mm culture tubes, cover with a 0.3 cm～0.4 cm liquid paraffin layer. Autoclave at 121℃ for 10 minutes. Final pH 7.2±0.1. Refrigerate, and discard if not used within 2 weeks. Immediately before use, steam or boil the base fluid for 10 min～15 minutes to remove free oxygen, cool quickly, and aseptically add 1.0 mL trypsin solution/15 mL broth.

Trypsin solution:

Trypsin(1 : 250)	1.5 g
H_2O	100.0 mL

Dissolve the trypsin into H_2O. Sterilize by filtering through 0.45 μm Millipore.

A3 Anaerobic egg yolk agar

Agar base:

Yeast extract	5.0 g
Tryptone	5.0 g
Proteose peptone	20.0 g
NaCl	5.0 g
Agar	20.0 g
H_2O	1 000.0 mL

Autoclave at 121℃ for 15 minutes. Final pH 7.2±0.2.

Liver veal-egg yolk agar:

Wash 2 or 3 eggs with stiff brush, and drain. Soak eggs in 0.1% $HgCl_2$ solution for 1 hour. Drain $HgCl_2$ solution and replace with 70% alcohol, soaking for 30 minutes. Take out the eggs, crack aseptically, and discard the whites. Gather the yolk with syringe, place it in sterile container, and add equal volume of sterile physiological saline. Mix thoroughly, store at 4℃ for future use.

Preparation of medium:

To each 500 mL of the agar base(48℃~50℃), add 80 mL of egg yolk-NaCl suspension. Mix thoroughly and pour plates. Dry the plates for 2 days at room temperature, or 24 hours at 35℃. Discard contaminated plates and store the sterile plates in refrigerator.

A4 Gram stain

A4.1 Hucker ammonium oxalate crystal voilet

a)

Crystal violet(90% dye)	2.0 g
Alcohol(95%)	20.0 mL

b)

Ammonium oxalate	0.8 g
H_2O	80.0 mL

Mix a) and b). Put aside for 24 hours filter through coarse paper.

Gram iodine solution:

Iodine	1.0 g
Potassium iodide	2.0 g
H_2O	300.0 mL

Put potassium iodide into mortar, add iodine, macerate with pestle for 5~10 seconds, add 1 mL H_2O and macerate, then add 5 mL H_2O and macerate, and then add 10 mL H_2O and macerate. Contain this solution in a reagent vase, wash the mortar and pestle with H_2O, collect the rinse to dilute the solution to a total of 300 mL.

A4.2 Hucker contrast solution(mother solution)

Safranine	2.5 g
95% alcohol	100.0 mL

Add 10 mL mother solution into 90 mL H_2O.

A5 Crystal violet

A5.1 Crystal violet solution in thin alcohol

Crystal violet(90% dye)	2.0 g
95% alcohol	20.0 mL
H_2O	80.0 mL

A5.2 Ammonium oxalate crystal violet(Hucker) solution(see A2.1)

The above 2 solutions are regarded as stable morphological stains.

A6 Loeffler's methylene blue

a)

Methylene blue(90% dye)	0.3 g
95% alcohol	30.0 mL

b)

Diluted KOH(0.01%)	100.0 mL

Mix a) and b).

A7 Disinfectants

A7.1 Tincture of iodine

Potassium iodide	10.0 g
Iodine	10.0 g
70% alcohol	500.0 mL

A7.2 NaClO solution

NaClO	5.0～5.25 g
H_2O	100.0 mL

A8 Gel-phosphate buffer

Gelatin	2.0 g
Na_2HPO_4	4.0 g
H_2O	1 000.0 mL

Dissolve gelatin and phosphate in H_2O with gentle heat. Autoclave at 121℃ for 20 minutes. Final pH 6.2.

A9 Physiological saline(0.85%)

NaCl	8.5 g
H_2O	1 000.0 mL

Dissolve NaCl in H_2O. Autoclave at 121℃ for 15 minutes. Cool to room temperature.

A10 1 mol/L NaOH solution

NaOH	40.0 g

Dissolve NaOH in H_2O, dilute to 1 000 mL. Used for adjusting pH value of culture media.

A11 1 mol/L HCl solution

HCl(concentrated)	89.0 mL

Add H_2O, dilute to 1 000 mL.