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我国养猪业废弃物中四环素类、磺胺类抗生素及相关抗性基因污染研究进展

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摘要: 我国是世界养猪第一大国,生猪饲养量和猪肉产量均位居世界第一。养猪业每年所产生的粪便、废水中含有大量畜用抗生素及其代谢产物,使养猪业废弃物成为环境中重要的抗生素污染源之一,随之产生的抗性基因污染及传播问题也不容忽视。本文结合近年来国内外的研究数据,对我国养猪业废弃物中四环素类、磺胺类抗生素及其相关抗性基因的检测方法、污染状况及影响抗性基因传播的因素进行了分析,并基于控制我国养猪行业抗生素及抗性基因污染的目的,提出了今后的研究重点。

关键词: 抗生素;抗性基因;四环素;磺胺;猪粪;养猪废水
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Contamination of Tetracyclines, Sulfonamides and Corresponding Resistance Genes in the Waste from Chinese Pig Industry

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Abstract: China has the world's largest pig industry in the number of hogs and the amount of pork production. The swine manure and wastewater produced from the pig industry contain a number of veterinary antibiotics and their metabolites, which make the swine waste an important pollution source of antibiotics to the environment. The subsequent contamination and dissemination of antibiotic resistance genes (ARGs) cannot be overlooked. Based on the research data in recent years, the detection methods and pollution status of tetracyclines, sulfonamides and the corresponding ARGs, as well as the impact factors on the dissemination of ARGs in the Chinese pig industry, were summarized in this paper. Moreover, the focus of further research is also proposed for the purpose of controlling the contamination of antibiotics and ARGs caused by the Chinese pig industry.

Keywords: antibiotics; antibiotic resistance genes (ARGs); tetracyclines; sulfonamides; swine manure; swine wastewater

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基于预防疾病和促进生长的目的,抗生素被大量应用于动物养殖业中。在我国,抗生素的过量使用在养殖业中普遍存在[1]。据报道,2013 年我国抗生素使用量在 16.2 万 t 以上,其中有 52% 用于畜禽养殖业[2]。我国是全世界最大的猪肉生产国,2014 年我国猪肉产量 5 671 万 t,占我国所有肉类总产量的 66.4% ,养猪业也成为抗生素的“使用大户”。然而,经口服或注射所施用的抗生素仅有小部分能被动物吸收代谢,而约有 30% ~90% 以原形态或络合物形态随动物排泄物排出,使得养猪业废弃物成为环境中抗生素污染的重要来源[3]。排放到环境中的抗生素除对动、植物体存在直接的生物毒性外[4],还将给环境中的细菌带来选择压力,造成抗性细菌的积累及抗性基因(antibiotic resistance genes, ARGs)的传播。携带有 ARGs 的细菌最终可能通过食物链进入人体,引发难以治愈的细菌性疾病,给人类健康带来严重威胁。近年来,我国养猪业中抗生素及 ARGs 的污染及其对周边土壤、水体环境的影响日益受到关注。本文根据国内外最新的研究进展及我国养猪业现状,以最常用的四环素类和磺胺类抗生素及其 ARGs 为分析和总结对象,对我国养猪业废弃物中抗生素及 ARGs 的检测、污染状况及传播进行了综述,并指出今后的研究重点和方向。

1　养猪业废弃物中抗生素及 ARGs 的检测方法(Methods for detection of antibiotics and ARGs in swine waste)

养猪业废弃物(粪便、废水)成分复杂、干扰物质多,加大了抗生素的检测难度。因此通常采用一系列的前处理步骤,最大程度地去除基质中的干扰物质,同时保证抗生素的萃取效率及有效富集。这使得前处理成为养殖废弃物中抗生素检测的关键步骤,对于最终结果的准确性及灵敏度至关重要。对于废水样本,通常采用固相萃取技术(solid phase extraction, SPE),将废水通过吸附小柱,采用一系列清洗、选择性洗脱的方式达到富集、分离和纯化样本中抗生素的目的[5]。对于粪便及土壤样本,首先需要采用机械振荡、超声等方式使基质中有机质充分解离释放,再采用 SPE 进行抗生素的富集[6]。样本通过前处理之后,所得到的含有抗生素的富集溶液即可通过仪器进行检测。为达到多种抗生素同步检测的目的,最常采用高效液相色谱(high performance liquid chromatography, HPLC)对抗生素进行分离,检测器多采用灵敏度和选择性较高的质谱(mass spectrometry, MS)及串联质谱(MS/MS)[7-9]。作者通过改进的 SPE-HPLC/MS 方法,成功检测出养猪废水中 9 种常见的抗生素[10],并通过溶剂萃取、超声等前处理方法,对养猪废水固相、猪粪中抗生素的提取过程进行了优化,使回收率达到了理想范围,构建了一套适用于养猪业废弃物中抗生素的定性、定量检测方法体系[6,11]。

同抗生素的检测方法类似,ARGs 的检测也需要首先对其进行富集。所采用的富集方法通常分为传统的依赖细菌培养和不依赖细菌培养的方法,二者各有优缺点。依赖细菌培养的方法采用含有抗生素的培养基选择性纯化培养带有抗生素抗性的细菌,再通过分子生物学方法分析菌株中的 ARGs 的基因型及其定位,该方法优势在于可以直接得到特定菌株的抗性基因型信息[12-14],但自然界中只有少数细菌生物是可培养的,因此导致依赖细菌培养的检测方法具有很大的局限性[15]。不依赖于细菌培养的方法,即通过直接提取环境样本中的 DNA[16-19],或者通过细菌筛选富集获得细菌群体[20],再以分子生物学方法对样本进行分析,这一方法虽然无法得知 ARGs 的具体宿主来源,但由于可以获得样本的总体数据,因此目前被广泛应用于环境样本中 ARGs 的定性和定量检测。由于养猪粪便、废水中含有大量未消化的有机质,故需采用有效的方法予以去除,否则会严重影响后续的生物学分析。因此,在提取样本总 DNA 时,多采用针对性强的 DNA 提取试剂盒来获得高纯度的样本总 DNA[16,21-23];对于细胞筛选,则应首先采用恰当方法对环境样本中的细菌进行分离纯化后用于下一步分析[20]。而检测 ARGs 所常用的分子生物学方法包括有聚合酶链反应(polymerase chain reaction, PCR)、探针杂交、实时荧光定量 PCR 及新一代 DNA 测序技术等。

1.1　PCR、Southern blot 和 DNA 芯片技术

PCR 技术以其简便快速、特异性强等特点,在 ARGs 的定性检测方面得到了广泛应用[24-25]。Wu 等[16]使用 PCR 方法在我国北京、天津、浙江等地的 9 家养猪场周边土壤中检出了 15 种常见的四环素类抗性基因(tetracycline resistance genes, TRGs);Barkovskii 和 Bridges[26]使用 PCR 方法在美国 3 家养猪场的粪便、土壤、废水样本中检出了 14 种 TRGs。此外,改进的 PCR 方法,如 multiplex PCR、nested-PCR

等,能够大幅度提高 PCR 的使用范围和精确度,达到快速定性检测 ARGs 的目的[27-28],因此也被用于畜禽养殖相关样本中 ARGs 的检测。Garofalo 等[29]利用 nested-PCR 技术,直接检测了鸡肉、猪肉及动物粪便中的 11 种 ARGs;Khan 等[30]利用 multiplex PCR 技术,在从牛奶、鸡舍废物中分离得到的肠球菌中检出了万古霉素类 ARG(*van*C1)。

具有一定同源性的核苷酸序列在一定条件下可以按碱基互补配对原则特异性地杂交形成双链,Southern blot 即是基于这一原理,通过设计并合成特异性 DNA 探针,可以定性地检测与探针 DNA 序列互补的片断。将 Southern blot 技术配合 PCR 方法使用,可使得到的结果可信度更高。Heuer 和 Smalla[19]采用该方法在猪粪施肥土壤中检出了 3 种磺胺类抗性基因(sulfonamide resistance genes, SRGs);Moura 等[31]通过此方法检测了屠宰场污水处理系统中的整合子(integrons)相关基因。

DNA 芯片技术(DNA microarray)是高密度、高通量的分子杂交检测技术,其克服了传统 Southern blot 操作繁琐、耗时长的缺点,可以在短时间内同时检测多种基因。Perreten 等[32]通过微阵列技术成功检测了革兰氏阳性菌中 90 种 ARGs;Frye 等[33]则更进一步,将该技术扩展到了革兰氏阴性菌,并认为通过该技术可以检测所有抗性细菌的 ARGs。综上所述,PCR 结合相关 DNA 同源性杂交检测技术可以达到对于基因的准确定性,但不能对序列同源性较近的基因进行准确的区分,只能进行粗略的半定量,因此在大多数情况下只能作为快速定性的方法。

1.2 实时荧光定量 PCR 技术

实时荧光定量 PCR 技术(Real-time PCR)是一种在普通 PCR 反应体系中加入荧光基团,利用荧光信号积累实时监控整个 PCR 进程,最后通过内参基因或标准曲线对未知模板进行定量分析的方法,它不仅实现了对 DNA 模板的定量,而且灵敏度更高、特异性和可靠性更强,使之在 ARGs 的定量检测方面得到广泛使用。国内外学者采用该方法,对猪、牛、鸡等多类畜禽养殖相关样本(包括养殖废水、厌氧塘沉积物、粪便、堆肥以及施肥土壤等)中的 TRGs 和 SRGs 的分布进行了定量检测[16-18,23,34]。

1.3 新一代 DNA 测序技术

近年来,基于焦磷酸测序技术[35-36]及循环芯片测序策略(cyclic-array sequencing)的新一代 DNA 测序技术趋于成熟[37]。该技术通过在芯片上同时运行的数百万个测序反应,得到大量的长度在几十到几百个碱基对范围的短序列,再将短序列拼接组装从而得到完整的样本基因组序列信息。与传统 Sanger 测序技术相比,新一代测序技术具有成本更低、数据量更大、信息更全面等优点,在 ARGs 的定性、定量检测方面已有一些应用[38-41]。同时,定性、定量 PCR 及探针杂交方法仅能检测已知 ARGs,而新一代测序技术则能通过功能宏基因组学分析寻找到新的潜在 ARGs[42]。因此,新一代测序技术将会在未来 ARGs 的分析研究中发挥重要作用。

2 我国养猪业废弃物中四环素类及磺胺类抗生素的污染现状(Contamination of tetracyclines and sulfonamides in the waste from Chinese pig industry)

有关检测数据显示,我国猪粪样本中四环素类抗生素的检出浓度多在 1~100 mg·kg^{-1} 浓度范围[43-47],与奥地利、丹麦等欧洲国家猪粪样本中的检出值(<46 mg·kg^{-1})相比稍高[48-49];磺胺类抗生素的检出浓度范围多在 0.1~10 mg·kg^{-1} 浓度范围[50-51],同国外检出数据基本持平[49,52]。山东是我国养殖业规模较大的省份,Pan 等[11]选取了山东 21 家典型集约化养猪场,采集并分析了 126 个猪粪样本的抗生素浓度,发现四环素类检出值和检出率均高于其他种类抗生素,检出率在 84.9%~96.8% 之间,其中金霉素的检出浓度最高,达到了 764.4 mg·kg^{-1},是目前为止四环素类抗生素在猪粪中检测到的最高浓度;磺胺类抗生素的检出率在 0.9%~51.6% 之间,其中磺胺二甲嘧啶检出浓度最高,达到 28.7 mg·kg^{-1}。

由于我国养猪场多采用水冲粪工艺,致使养猪场废水中抗生素的检出率和检出浓度也较高,并可随废水排放迁移至邻近地表水中。Wei 等[53]对江苏省 21 家养猪场的废水及邻近河流水进行了分析,发现在废水中四环素类和磺胺类是检出率最高的抗生素,最高浓度分别达到了在 72.9 μg·L^{-1}(土霉素)和 211 μg·L^{-1}(磺胺二甲嘧啶),在河流水中 2 类抗生素的最高检出浓度也分别达到了 2.42 μg·L^{-1}(金霉素)和 4.66 μg·L^{-1}(磺胺二甲嘧啶)。Tong 等[54]检测了武汉市 2 家养猪场废水中的四环素类、磺胺类抗生素,发现磺胺甲嘧啶和土霉素的浓度较高,最高检出浓度均超过 10 μg·L^{-1}。Ben 等[10,55]对北京、山东共 20 余家养猪场的废水进行了常用抗生素的浓度测定,结果显示北京地区样本中磺胺类和四环素类的最高检出浓度分别达到 14.05 μg·L^{-1}(磺胺间二甲氧嘧啶)和 32.67 μg·L^{-1}(金霉素),在山东地区的样本中,

磺胺二甲嘧啶、土霉素和金霉素的浓度较高，三者的检出中值浓度分别为 14.56、8.05 和 6.01 $\mu g\cdot L^{-1}$，其中土霉素的最高检出浓度达到了 2.02 $mg\cdot L^{-1}$。以上数据同国外检出数据相比，大致处于同一数量级[56-57]。

养猪业所产生的粪便和废水中残留的抗生素可通过施肥、灌溉、非人为扩散等多种方式向土壤中迁移。四环素类及磺胺类抗生素在我国土壤中的残留现象较为普遍。在我国北方采集的施肥土壤样本中，2 类抗生素的最高检出浓度分别为 2 683 和 32.7 $\mu g\cdot kg^{-1}$，同时研究显示，由于施肥多在冬季进行，导致冬季土壤中的抗生素检出浓度远高于夏季[58]。在珠江三角洲地区采用猪粪施肥的菜田土壤中，2 类抗生素的最高检出浓度分别为 242.6 和 321.4 $\mu g\cdot kg^{-1}$[59]；在从福建沿海多个城市农田土壤中的浓度分析结果显示，四环素类抗生素的最高检出浓度也达到了 2 669 $\mu g\cdot kg^{-1}$[60]。以上报道所调查的浓度数据同国外数据相比处于同一水平，但最高检出浓度略高于国外[61-62]。

3　我国养猪业废弃物中 TRGs 和 SRGs 的污染特征、传播及影响因素（Contamination and dissemination of TRGs and SRGs and related impact factors in the waste from Chinese pig industry）

细菌对四环素类抗生素的抗性机制主要分为 3 种[63-65]：核糖体保护机制(ribosomal protection proteins, RPP)，如 *tet*M、O、Q、S、T、W 等；外排泵机制(efflux pumps proteins, EFP)，如 *tet*A、B、C、G、K、L 等；酶学修饰机制(enzymatic inactivation, EI)，如 *tet*X 等。细菌对磺胺类抗生素的抗性机制主要是靠获得表达产物可以避免磺胺类抗生素侵害的二氢叶酸合成酶(dihydropteroate synthase, DHPS)突变基因，主要指 *sul1*、*sul2* 和 *sul3*，其中 *sul1* 和 *sul2* 是环境中存在最为普遍、丰度较高的 SRGs[64]。

近年来关于我国养猪废弃物中 ARGs 的污染情况也有所报道。从现有调查数据可知，猪粪、废水中含有较高浓度的 TRGs 和 SRGs，且不同地域间的差别较小。各类 TRGs 的相对丰度(ARG 与 16s rDNA 丰度的比值)略有差异，粪便中 RPP-TRGs 和 EI-TRGs 通常较高，约在 $10^{-4}\sim10^{-1}$之间，EFP-TRGs 则相对较低，相对丰度约在 $10^{-5}\sim10^{-3}$左右[34,66]；废水中 3 种 TRGs 的水平基本持平，均在 $10^{-4}\sim10^{-1}$范围[34]。SRGs(*sul1* 和 *sul2*)在粪便中的相对丰度在 $10^{-4}\sim10^{-3}$左右，而废水中的丰度可达 $10^{-2}\sim10^{-1}$[34]。在土壤中，TRGs 和 SRGs 丰度约在 $10^{-5}\sim10^{-2}$范围波动，与养猪废弃物的施用方式密切相关[16,67]。

ARGs 在环境中的传播扩散除靠抗性细菌的自身繁殖外，还借助于各种基因水平转移方式，包括细菌之间的质粒接合转移、噬菌体介导的转导作用、及细菌直接摄取裸露 DNA 从而获得 ARGs 的自然转化作用[68-70]。这 3 种方式中，噬菌体转导具有高度的宿主特异性，使 ARGs 的转移限制于同种细菌间；自然转化作用在环境中的发生具有一定的随机性，需要依靠具有天然转化能力的受体细菌，而此类细菌种类稀少，同时游离 DNA 稳定性差，增加了 ARGs 通过自然转化作用传播的局限性；相比而言，质粒的宿主范围广，通常含有多种 ARGs，并且质粒的接合转移是细菌之间基因交流的主动方式，加之质粒中通常含有其他能够介导基因获取及转移的相关基因元件从而促进 ARGs 的传播，使之成为环境中介导 ARGs 水平转移的主要方式[71-72]。猪粪、养猪废水是抗性质粒的重要载体，已有研究报道表明其中含有多种类型的、可在细菌间传播的抗性质粒[73-74]。当这些废弃物通过排放、灌溉、施肥等不同途径与环境水体或土壤接触时，会将携带有 ARGs 的抗性细菌带入这些环境介质中，并通过质粒及其他基因元件如整合子[19,34]、转座子[17,75]等，促进其中 ARGs 的扩散[76-78]。

养猪业废弃物(粪便、废水)通常存在较高浓度的抗生素残留，并且由于持续排放，使其在被污染的土壤、水体中逐渐累积，直接造成抗性选择压力导致环境中相关抗性细菌及 ARGs 的增殖。Heuer 和 Smalla[19]将含有磺胺嘧啶的猪粪施肥于土壤，发现其中 *sul1* 基因的丰度在至少 2 个月内有所上升；在畜禽废水中，SRGs 的相对丰度与磺胺类抗生素的残留浓度呈现较强的相关性[18]；我国学者对浙江、北京、天津、福建沿海等地养猪场周边的土壤样本的分析结果显示，其中所含有 TRGs 的相对丰度与四环素类抗生素的残留浓度也存在着显著正相关性[16,60]。同时，一种抗生素的存在也可以影响其他非针对于此抗生素的 ARGs[18]，这可能与细菌多重抗药性相关[41]。除抗生素之外，粪便、废水中含有的常规污染物，如 COD、氮、磷等，也对于 ARGs 的积累有促进作用[18,79]；一些重金属元素与抗生素存在抗性共选择效应，因此这些金属元素的存在也对于 ARGs 的积累起到了促进作用[17-18]。水分是维持细菌生命活动正常进行的最根本条件，干燥条件会使

携带有 ARGs 的细菌失水死亡[80]。温度也对 ARGs 有一定影响,低温(0～5 °C)可显著延长携带有 ARGs 的细菌在环境中的存活时间从而有利于 ARGs 的驻留[80];高温则有助于消减 ARGs,例如高温堆肥不仅可以消减粪便中的抗生素[81-87],对 ARGs 也有较为明显的消减效果[21,88-89]。另外,日照也是影响 ARGs 丰度水平的因素之一,据报道在养殖废水处理塘中,日照时间与其中的 TRGs 丰度呈现负相关[23,90]。

4 结论与展望(Conclusions and prospects)

由于技术、经济和管理等的局限性,我国大多数养猪场废弃物并未得到妥善处理,废水目前仍以直排为主,而粪便则多采用简单室内堆放风干处理,仅少数进行堆肥或发酵处理。鉴于养猪废弃物所造成的抗生素及 ARGs 污染十分值得关注,针对此问题,本文提出如下建议:

(1)应针对污染物从养殖源(动物肠道、粪便、养殖废水等)到环境介质(土壤、水体等)的排放途径,深入研究抗生素的环境行为、降解途径、机理及产物,以及 ARGs 的演化、扩散规律及关键影响因素。

(2)通过对养猪粪便、废水及施肥土壤中 ARGs 的种类分布与丰度的调查,得到 ARGs 的生态毒理基础数据,结合目前一些已知有效的控制抗生素及 ARGs 的方法,建立起一套适应我国国情的养猪废弃物控制策略,从而更好的控制 ARGs 在环境中的扩散,促进我国养猪业合理、健康的发展。

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