Identification of pathogens in the groundwater of laying hen flocks and selection of sensitive drugs

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Received: December 18, 2016; accepted: March 23, 2017.

To identify the microorganisms polluting the groundwater in a laying hen flock in South Henan province and select sensitive drugs, the current study evaluated the total bacterial counts and performed bacterial biochemical tests, gene sequencing, phylogenetic tree construction and sensitive strain screening tests. The total number of bacteria in the groundwater was 3.48×10^4 CFU/ml. The microorganism causing the groundwater pollution was Escherichia coli, which was highly sensitive to enrofloxacin. Therefore, emission of livestock manure should be given attention by the breeding enterprises, so as not to pollute the groundwater of the farm and endanger the health of livestock and poultry.

Keywords: groundwater; pathogens; sensitive drug.

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Introduction

In recent years, the livestock was the most active growth point and main pillar industry in Chinese rural economy, and it was also an important way and source for farmers to get rid of poverty. But this rapid development is mainly the traditional mode of livestock and poultry farming. In this mode, the development of the livestock itself was emphasized, the production and economic benefits were pursued excessively. The harmonious development of aquaculture and the surrounding environment was neglected.

The pollution was caused by livestock and poultry without harmless-treated, it was directly discharged into the water, soil, and air. The environment was deteriorated in the place, human and animal could not normal life [1].

If the soil of the farm was polluted by the excreta of microorganisms, the pathogens of the soil were further spread and ground water was also contaminated. Water-borne Salmonella and E. coli were the main force of microbial contamination of groundwater [2, 3, 4, 5].

The hens in a laying hen flock in South Henan province were experiencing enteritis or intestinal syndrome. The syndrome had a long shapeless stool and diarrhea. The hens easily relapsed after post-treatment drug withdrawal. After the exclusion of other factors, we determined that the etiologic factor might be groundwater pollution. Therefore, the drinking water of the laying hen flock was tested using biological diagnostic methods to determine the species and quantity of microorganisms in the drinking water. This research could improve the awareness of livestock farms to the pollution of

livestock manure. Finally, it would achieve the purpose of healthy breeding of livestock and poultry.

Materials and methods

Ground Water Samples

Three houses were randomly selected in the hens breeding farms, one row was randomly selected in each henhouse. Each water line is divided into three segments evenly by 2 points. Water was sampled at the 2 points. a total of six sampling points, respectively, labeled as I, II, III, IV, V, and VI. The water samples were collected to determine the total bacterial counts in the drinking water.

Well water samples

The water intake was collected from the layer groundwater well, 8 meters from the farmland irrigation wells, 14 meters from the farmland irrigation wells, and 25 m from the farmland irrigation wells, which use motor-pumped well water. The sites were denoted A, B, C and D, respectively. The depths of the four wells were 30 m-35 m. The water samples were collected to determine whether the groundwater was polluted by fecal sewage.

Sampling was performed according to the standard method. The water samples were sent back to the laboratory as soon as possible. The total number of bacteria in the water sample was measured by using the methodology described in GB 18918-2002.

Colony counts

The water samples were diluted in equal proportions (10^0, 10^{-1}, 10^{-2}, 10^{-3}, and 10^{-4}) with sterile water and spread onto nutrient agar solid medium. Three parallel tests were performed on each sample. The samples were placed in a 37°C constant temperature and humidity incubator for 24 h to observe colony formation. The colonies were counted thereafter [6].

Bacterial morphological observations

The bacteria in the smear were selected from the dominant colony. The bacterial morphology was observed by Gram staining [6].

Biochemical testing of the dominant colonies

The dominant colonies were separated and purified. Bacteria were assessed based on the decomposition of sugar and tryptophan and the carbon source selection [6].

16S rDNA sequence determination and phylogenetic tree construction

Total DNA was extracted from the strain as a template for PCR amplification of the 16S rDNA sequence by using the 27F (5'-AGAGTTTGATCMTGGCTCAGC-3') and 1492R primers (5'-GGTTACCTTGTTACGACTT-3'). The reaction conditions were as follows: pre-degeneration at 98°C for 5 min, 35 cycles of 95°C for 35 s, 55°C for 35 s, and 72°C for 90 s, followed by extension for 8 min. After gel electrophoresis, the PCR product was inserted into the pMD18-T vector and transformed into *E. coli* competent cells DH5α. The correct insertion of the fragment was determined by sequencing at the Nanjing Jinsirui Biotechnology Co. Ltd. The 16S rDNA sequencing results were submitted to the GenBank nucleic acid sequence database for gene alignment. The phylogenetic tree was constructed using the neighbor-joining method by MEGA 6.0 with a bootstrap stability test.

Sensitive drug screening

The broth culture precipitates of the dominant bacteria were concentrated to 5 µl, evenly coated onto nutrient agar medium, and treated with drug sensitivity tablets. The diameter of the inhibition zone on the medium was measured by using a Vernier caliper after 12 hours.

Data Presentation and Statistical Analysis

The SPSS software (19.0) was used in the research for quantitative analyses of the data. All data were expressed as mean ± SEM.

Table 1. The total numbers of bacteria in the water samples from the layer farm (unit: 10^4 cfu/ml).

Water Samples	I	II	III	IV	V	VI
1	3.49	3.47	3.42	3.44	5.32	3.42
2	3.47	3.42	3.38	3.50	3.46	3.47
3	3.42	3.40	2.30	3.52	3.42	3.40
Mean ± Standard Deviation	3.46±0.04	3.43±0.04	3.03±0.64	3.49±0.04	4.07±1.09	3.43±0.04

Table 2. Total bacterial counts in the four water wells (unit: 10^4 cfu/ml).

Water Samples	A	B	C	D
1	3.17	0.0086	0	0
2	3.32	0.0085	0	0
3	3.26	0.0076	0	0
Mean ± Standard Deviation	3.25±0.075	0.0082±0.0006	0	0

Table 3. The biochemical test results of the strain.

Bacterium	I	II	III	IV	V	VI
Glucose Fermentation Test	⊕	⊕	⊕	⊕	⊕	⊕
Lactose Fermentation Test	⊕	⊕	⊕	⊕	⊕	⊕
Maltose Fermentation Test	⊕	⊕	⊕	⊕	⊕	⊕
Mannitol Test	⊕	⊕	⊕	⊕	⊕	⊕
Sucrose Fermentation Test	-	-	-	-	-	-
Indole Test	+	+	+	+	+	+
Methyl Red Test	+	+	+	+	+	+
Voges-Proskauer Test	-	-	-	-	-	-
Citric Acid Utilization Test	-	-	-	-	-	-
Trisaccharide Iron Agar Test	-	-	-	-	-	-

⊕: produces acid and gas, +: produces acid but not gas, -: does not produce acid and gas.

Results

Determination of the total number of bacteria in the water samples

The number of bacteria in the water samples was obtained by partitioning (Table 1). The total number of bacteria in the water samples at the 6 sampling points far exceeded the national limit of 100 CFU/ml. The total number of bacteria in the groundwater was 3.48×10^4 CFU/ml.

The numbers of bacteria in the water samples from the four wells were obtained by partitioning (Table 2). The groundwater of the laying hens was seriously polluted, and the nearby farmland irrigation wells were contaminated with bacteria. Conversely, the distant farmland irrigation wells were not contaminated with bacteria.

Colonies and bacterial morphological observations

The colonies with round, convex, smooth surfaces, a gray-white color, a neat edge and a 2-3 mm diameter were the dominant colonies at the 6 sampling sites. The bacteria of the dominant colonies from the 6 sampling points were examined by Gram staining. All the bacteria were Gram negative rods that were 0.5-1.5 nm in length.

Biochemical test results

Biochemical tests were performed with the dominant colonies from the six sampling points. The results are shown in Table 3. The bacteria of the dominant colonies in the 6 sampling sites had the same ability to decompose sugar and tryptophan and used the same carbon source.

The six strains were the same bacterium according to the colony morphology, bacterial morphology, and biochemical test results.

Sequencing and phylogenetic analysis of the bacteria

The 16S rDNA sequence of HNZMD01 was 1,395 bp in size. The 16S rDNA sequencing results of HNZMD01 were compared with the NCBI database, the selected sequences were arranged using the Clustalw program in MEGA 6.0, and the phylogenetic tree was constructed using the Neighbor-joining (NJ) method (Figure 1). The homology of HNZMD01 with *Escherichia coli* (KU161315.1, KU161312.1, KM372219.1 and KC985144.1) was as high as 99%. The phylogenetic tree showed that HNZMD01 had 81% self-test support on the same branch as *E. coli*, which indicated that HNZMD01 had the closest genetic distance to *E. coli*.

Drug sensitivity test results

The drug sensitivity test was performed with the aim of preventing and treating enteritis with common antibiotics. The antibiotic circle diameters are shown in table 4. Norfloxacin hydrochloride was the only sensitive drug (circle diameter greater than 15 mm) for the *E. coli* strain in the groundwater of the laying hens. Florfenicol was more sensitive (circle diameter greater than 12 mm), whereas neomycin, amoxicillin, doxycycline hydrochloride, and colistin were low-sensitivity drugs (circle diameters less than 10 mm) [6].

Discussion

Recently, the non-point source pollution of livestock and the intrusion of poultry manure sewage into water and soil have become serious concerns. These issues have been studied using various tests, including biological oxygen demand (BOD), chemical oxygen demand (COD), suspended solids (SS), total nitrogen (TN), and total phosphorus (TP) [7, 8, 9, 10]. However, the pollution of livestock and poultry drinking water contaminated by microorganisms from the feces of livestock and poultry that cause disease of livestock and poultry has rarely been reported [11, 12].

In the study, the total number of bacteria was measured by GB 18918-2002. It was a serious excessive (3.48×10^4 CFU/ml) comparing to the national limit of 100 CFU/ml. The same bacteria were demonstrated in 6 water samples by observing the bacterial colony, bacterial

Table 4. Drug sensitivity test results.

Serial Number	Medicine	Diameter Inhibiting Zone (mm)
1	Norfloxatin	22
2	Florfenicol	13.5
3	Neomycin Sulfate	7.8
4	Amoxicillin	6.2
5	Doxycycline Hyclate	4.1
6	Colistin	3.5

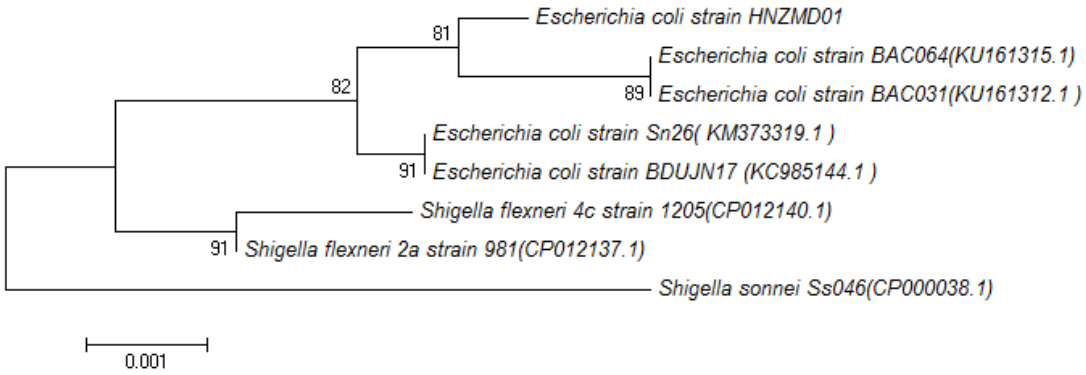

Figure 1. Phylogenetic tree of the 16S rDNA sequence of strain HNZMD01. The horizontal branch length represents the expected number of substitutions. The numbers of nodes are the bootstrap values (1,000 repetitions).

morphology and biochemical test results. It had been proven to be *E. coli* by the molecular biological method. According to the clinical symptoms of the hens, the pathogenic microorganism of the hens was *E. coli* in drinking water. *E. coli* was found in groundwater contaminated with livestock and poultry manure by Economides [3] and Zhang [13].

In this study, we analyzed the numbers of bacteria in the layer and surrounding groundwater. Based on the results, microorganisms (especially *E. coli*) in the sewage of livestock and poultry manure were detected in a surface water-soil water-groundwater system (SW-SoW-GW) in the groundwater, which in turn polluted groundwater sources [14]. Livestock and poultry manure sewage microorganisms can spread through surface water or soil water, resulting in pollution of surrounding livestock and poultry farms. Thus, a scale field that has been used to breed layers for 12 years is likely to cause groundwater bacterial contamination. The spread of *E. coli* in groundwater was also confirmed by Dwivedi et al. [5].

E. coli is facultative anaerobic bacterium that can survive for up to 1 month in humid, dark environments. The survival time of *E. coli* O157:H7 in soil was reported by Jiang et al. [15]. The results showed that *E. coli* survived for 231 days in sterilized soil and 193 days in non-sterilized soil [15]. These characteristics enable *E. coli* to easily pollute farm water. This pollution can be persistent pollution and is extremely

difficult to recover. On this farm, *E. coli* caused a long-term, continuous invasion of the laying hen field. By drinking contaminated groundwater, the laying hens developed enteritis or enterotoxic syndrome because the persistence of the pathogens contributed to ineffective treatment or relapse after drug withdrawal.

The *E. coli* in the drinking water was not sensitive to neomycin, amoxicillin, doxycycline hydrochloride, or colistin, which might explain why the long-term use of these drugs to prevent diseases failed in the field. Pollution of domestic water by microbes in farm waste water can occur through surface runoff or the groundwater system. The water most often causes diarrhea and other digestive problems. Therefore, the selection of medicine should be undertaken with prudence [15, 16].

To ensure the healthy and orderly development of breeding livestock, the safety of the drinking water should be regularly monitored. New farms must provide a harmless treatment and should use an impermeable floor layer to prevent and control manure sewage infiltration into the groundwater and to prevent pathogenic microorganisms from entering the drinking water system.

Acknowledgments

This work was supported by Key Research Projects of Higher Education in Henan Province (16B230005).

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