



Rapid detection of *Klebsiella pneumoniae*, *Klebsiella oxytoca*, *Raoultella ornithinolytica* and other related bacteria in food by lateral-flow test strip immunoassays

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ABSTRACT

Bacteria of the genera *Klebsiella* and *Raoultella*, which are present in foods and the natural environment, are associated with health hazards in humans. In the present study, two types of strips-based methods were developed to detect these bacteria simply and quickly. One method used lateral-flow test strips (LFTS) in combination with anti-*Klebsiella* antibodies labeled with palladium nanoparticles that bind to target bacteria, allowing their visualization. In the other, the antibodies were immobilized on nitrocellulose membranes, and urease activity was measured using an ion sensitive field effect transistor pH sensor. Testing of a combination of these two methods on 72 cultured strains successfully identified all 25 strains of *Klebsiella pneumoniae*, *Klebsiella oxytoca* and *Raoultella ornithinolytica*. This approach also accurately identified 76 of 77 (99%) strains isolated from meats and pastries. When combined with preculture, this method was accurate in identifying 19 of 26 (73%) target bacteria in food. These results suggest that the novel combination of strip-based assays may be effective for the on-site monitoring of food production plants, and thereby enhance food safety.

1. Introduction

The bacterial genera *Klebsiella* and *Raoultella* are opportunistic pathogens belonging to the family *Enterobacteriaceae* (Brisse et al., 2006; Sękowska, 2017). These bacteria, primarily *Klebsiella pneumoniae* and, to a lesser extent, *Klebsiella oxytoca* and *Raoultella ornithinolytica*, are responsible for various public health problems, including respiratory and urinary tract infections, liver abscesses and septicemia (Brisse et al., 2006; Sękowska, 2017). *K. pneumoniae* is the second most frequent cause of nosocomial Gram-negative bacteremia (Podschun and Ullmann, 1998). Recently, the spread of *Klebsiella* strains producing extended-spectrum β -lactamase (ESBL) and carbapenemase has become a major public health problem (Legese et al., 2017; Upadhyay et al., 2017), because these enzymes are often encoded by mobile genes and are easily transferred to closely related species, resulting in antibiotic resistance (Brisse et al., 2006).

K. pneumoniae, *K. oxytoca* and *R. ornithinolytica* are ubiquitous in nature and are often detected in foods, sewage, soil, plants and the gastrointestinal tracts of animals (Brisse et al., 2006; Sękowska, 2017). *R. ornithinolytica* has been isolated from mackerel (Kanki et al., 2002). *R. ornithinolytica* produces histamine and is a cause of mackerel food poisoning. *K. pneumoniae* has been isolated from meats, vegetables and pastries (Babu et al., 2013; Falomir et al., 2013; Kato et al., 2014), and

Klebsiella strains with virulence properties, including the presence of siderophores, serum resistance, and the production of hemolysin and ESBL, have been detected in beef and chicken (Gundogan et al., 2011). Because of their habitats and characteristics, these bacteria are considered reservoirs of antibiotic-resistance genes in the food chain (Kim et al., 2005). Therefore, it is necessary to monitor foods and the environment for *K. pneumoniae* and related bacteria (Gundogan et al., 2011; Upadhyay et al., 2017).

Bacterial detection generally depends on phenotype or genotype. A conventional phenotype-based method is culturing (Legese et al., 2017). Suspended food in buffer solution or a sample from the environment is applied to a medium, such as a nutrient agar plate. Resultant colonies are picked up and subcultured, and their utilization of carbon sources and the molecules that act as enzyme substrates are investigated, resulting in the identification of bacterial species. Although regarded as the gold standard, culture methods are time-consuming (Gupta et al., 2015). In contrast, genotype-based methods are rapid. In these methods, a specific region of genomic DNA is PCR amplified, and the amplified sequence is visualized with a fluorescent dye (qPCR) or by its electrophoresis pattern, leading to the detection of a target bacterium (Almeida et al., 2017; Gokduman et al., 2016; Rawool et al., 2016; Tominaga, 2006; Tominaga, 2007). Although rapid and highly sensitive, PCR-based methods require dedicated devices, making

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them inappropriate for testing on site (Shan et al., 2015).

More recently, bacteria have been detected by lateral-flow test strip (LFTS) assays (Dzantiev et al., 2014; Kim et al., 2015; Ramos et al., 2017; Tominaga, 2017). In this method, bacterial cells in solution are mixed with antibody labeled with colloidal gold or palladium nanoparticles (PdNPs) and applied to a test strip. These complexes migrate on the test strip by capillary action and are captured by another antibody fixed on the strip in advance, yielding a colored line or spot derived from aggregated metal colloid, thereby detecting bacteria. The time required for this assay is only about 15 min, and no special equipment is required.

This study describes the development of LFTS assays to detect bacteria in the *Klebsiella* group (*K. pneumoniae*, *K. oxytoca* and *R. ornithinolytica*). The strains were detected within 15 min without requiring special equipment. Combining this assay with measurements of urease activity improved the accuracy of detection. This method could be applied to food production to prevent the spread of *Klebsiella* in foods.

2. Materials and methods

2.1. Bacterial strains and growth conditions

The strains used in this study are shown in Table 1. Bacteria were cultured in LB medium (Becton, Dickinson and Company, Franklin Lakes, NJ, USA) with shaking at 37 °C for 16–20 h. To make LB plates, 2% (w/v) agar was added to the medium. Culture liquid was appropriately diluted by peptone-containing physiological saline solution and streaked on the plate. After 16–20 h at 37 °C, emergent colonies were counted and the density of bacteria in the culture liquid was estimated.

2.2. Development of LFTS

LFTS was constructed as described (Tominaga, 2017). Briefly, nitrocellulose membranes (HiFlow Plus HFB07504), cellulose fiber absorption pads, and glass fiber pads were obtained from Millipore Corporation (Darmstadt, Germany). PdNP solutions were purchased from Winered Chemical Corporation (Tokyo, Japan). A nitrocellulose membrane was cut into strips (5 mm wide, 25 mm long), an absorption pad (20 mm wide and 10 mm long) was placed downstream of the membrane, and a glass fiber pad (5 mm wide and 10 mm long) was placed upstream of the membrane. Purified polyclonal antibodies (pAbs) to the genus *Klebsiella* were immobilized onto a nitrocellulose membrane (0.08 µg/test; capture pAbs). The same pAbs were also labeled with PdNPs (1 µg/test; detection pAbs). Bacterial cells were collected from culture solution by centrifugation at 9600g for 1 min, and then washed with physiological saline and heated for 15 min at 100 °C. These cells were mixed with detection pAbs and applied to a glass fiber pad. Images of the test strips were acquired with a scanner MX 893 (Canon, Tokyo, Japan) at a resolution of 600 dpi, and the density of each spot was quantified (signal values: a.u.). Signals of > 800, 400–800, and < 400 a.u. were scored as (+ +), (+), and (–), respectively.

2.3. Measurement of urease activity

Approximately 10⁸ bacterial cells were applied to a nitrocellulose membrane strip, on which the capture pAbs had been immobilized. The site of antibody immobilization was excised, with 1.5 mm width, and the two pieces were each incubated in 40 µL of 1% urea solution for 3 h. The resultant pH was measured using an Ion Sensitive Field Effect Transistor (ISFET) semiconductor pH sensor, S2K922 (ISFETCOM, Saitama, Japan). Samples showing a ≥1 increase in pH value were defined as those positive for urease activity (+).

Table 1

LFTS assay results of the cultured bacterial strains.

		LFTS assay
<i>Klebsiella pneumoniae</i>	IAM 1063	++
<i>Klebsiella pneumoniae</i>	NBRC 3512	++
<i>Klebsiella pneumoniae</i>	NBRC 13277	++
<i>Klebsiella pneumoniae</i>	NBRC 13541	++
<i>Klebsiella pneumoniae</i>	NBRC 13703	++
<i>Klebsiella pneumoniae</i>	NBRC 14440	++
<i>Klebsiella pneumoniae</i>	NBRC 14442	++
<i>Klebsiella pneumoniae</i>	NBRC 14443	++
<i>Klebsiella pneumoniae</i> subsp. <i>pneumoniae</i>	NBRC 14940 ^T	++
<i>Klebsiella pneumoniae</i> subsp. <i>rhinoscleromatis</i>	NBRC 105682 ^T	++
<i>Klebsiella pneumoniae</i> subsp. <i>ozaenae</i>	NBRC 105683 ^T	++
<i>Klebsiella pneumoniae</i>	NBRC 3318	+
<i>Klebsiella pneumoniae</i>	NBRC 3321	+
<i>Klebsiella pneumoniae</i>	NBRC 12059	+
<i>Klebsiella pneumoniae</i>	NBRC 12009	+
<i>Klebsiella pneumoniae</i>	NBRC 12932	+
<i>Klebsiella pneumoniae</i>	NBRC 14438	+
<i>Klebsiella pneumoniae</i>	NBRC 14441	+
<i>Klebsiella oxytoca</i>	ATCC 8724	++
<i>Klebsiella oxytoca</i>	ATCC 43086	++
<i>Klebsiella oxytoca</i>	ATCC 43165	++
<i>Klebsiella oxytoca</i>	ATCC 43863	++
<i>Klebsiella oxytoca</i>	ATCC 49131	++
<i>Klebsiella oxytoca</i>	ATCC 700324	+
<i>Raoultella ornithinolytica</i>	JCM 6096 ^T	++
<i>Serratia grimesii</i>	NBRC 13537 ^T	++
<i>Enterobacter aerogenes</i>	IAM 12348 ^T	+
<i>Enterobacter aerogenes</i>	NBRC 12010	+
<i>Enterobacter cloacae</i> subsp. <i>cloacae</i>	IAM 12349 ^T	+
<i>Enterobacter cloacae</i> subsp. <i>cloacae</i>	NBRC 13536	–
<i>Escherichia coli</i>	NBRC 102203 ^T	+
<i>Escherichia coli</i>	NBRC 3972	–
<i>Cedecea davisae</i>	NBRC 105702 ^T	–
<i>Citrobacter braakii</i>	ATCC 43162	–
<i>Citrobacter freundii</i>	IAM 12471 ^T	–
<i>Citrobacter koseri</i>	NBRC 105690	–
<i>Cronobacter sakazakii</i>	JCM 1233 ^T	–
<i>Enterobacter hormaechei</i>	NBRC 105718 ^T	–
<i>Erwinia aphidicola</i>	NBRC 102417 ^T	–
<i>Erwinia persicina</i>	NBRC 102418 ^T	–
<i>Escherichia albertii</i>	NBRC 107761 ^T	–
<i>Escherichia fergusonii</i>	NBRC 102419 ^T	–
<i>Escherichia hermannii</i>	NBRC 105704 ^T	–
<i>Escherichia vulneris</i>	JCM 1688 ^T	–
<i>Escherichia blattae</i> → <i>Shimwellia blattae</i>	NBRC 105725 ^T	–
<i>Hafnia alvei</i>	JCM 1666 ^T	–
<i>Kluyvera ascorbata</i>	IAM 14203 ^T	–
<i>Kluyvera cryocrescens</i>	NBRC 102467 ^T	–
<i>Kluyvera intermedia</i>	JCM 1238 ^T	–
<i>Leclercia adacarboxylata</i>	ATCC 23216	–
<i>Leclercia adacarboxylata</i>	JCM 1667 ^T	–
<i>Lelliottia amnigena</i>	JCM 1237 ^T	–
<i>Pantoea agglomerans</i>	NBRC 12686	–
<i>Pectobacterium carotovorum</i> subsp. <i>carotovorum</i>	NBRC 103133	–
<i>Raoultella planticola</i>	JCM 7251 ^T	–
<i>Raoultella terrigena</i>	JCM 1687 ^T	–
<i>Serratia ficaria</i>	NBRC 102596 ^T	–
<i>Serratia fonticola</i>	JCM 1242 ^T	–
<i>Serratia liquefaciens</i>	JCM 1245 ^T	–
<i>Serratia marcescens</i> subsp. <i>marcescens</i>	NBRC 102204 ^T	–
<i>Serratia odorifera</i>	NBRC 102598 ^T	–
<i>Serratia plymuthica</i>	NBRC 102599 ^T	–
<i>Serratia rubidaea</i>	NBRC 103169 ^T	–
<i>Yersinia bercovieri</i>	NBRC 105717 ^T	–
<i>Yersinia enterocolitica</i> subsp. <i>enterocolitica</i>	ATCC 9610	–
<i>Yersinia enterocolitica</i> subsp. <i>enterocolitica</i>	ATCC 27729	–
<i>Yersinia enterocolitica</i> subsp. <i>enterocolitica</i>	ATCC 23715	–
<i>Yersinia rohdei</i>	NBRC 105715 ^T	–
<i>Yersinia ruckeri</i>	NBRC 102019	–
<i>Bacillus subtilis</i> subsp. <i>subtilis</i>	NBRC 13719 ^T	–
<i>Lactobacillus plantarum</i> subsp. <i>plantarum</i>	NBRC 15891 ^T	–
<i>Staphylococcus saprophyticus</i> subsp. <i>saprophyticus</i>	NBRC 102446 ^T	–

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