Rapid detection of methicillin-resistant *Staphylococcus aureus* in pork using a nucleic acid-based lateral flow immunoassay

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Abstract

Methicillin-resistant *Staphylococcus aureus* (MRSA) is considered as one of the leading causes of food poisonings worldwide. Due to the high prevalence and extensive challenges in clinical treatment, a rapid and accurate detection method is required to differentiate MRSA from other *S. aureus* isolated from foods. Since the methicillin resistance of *S. aureus* is due to the acquisition of the *mecA* gene from staphylococcal chromosome cassette, the presence of the *mecA* gene is interpreted as a marker for the identification of MRSA. In this study, a low-cost lateral flow immunoassay (LFI) strip was used to detect the *mecA* amplicons subsequent to polymerase chain reaction (PCR). The specificity of this PCR-LFI assay was tested between MRSA and methicillin-susceptive *S. aureus*. Both the test line and control line were shown up on the LFI strip for MRSA, whereas only the control line developed for methicillin-susceptive *S. aureus*. The detection limit of PCR-LFI assay was 20 fg for genomic DNA (100 times more sensitive than gel electrophoresis) and 2×10^0 CFU per 100 g of pork products after enrichment at 37°C for 48 h. The total detection time of using LFI was 3 min, which was faster than the conventional electrophoresis (~45 min). With the performance of PCR-LFI, 7 out of 42 *S. aureus* isolates were identified to be MRSA from imported pork products, which was consistent to the standardized minimum inhibitory concentration assay. This *mecA*-based PCR-LFI strip can be used for rapid and accurate detection of MRSA isolated from commercial pork products.

Keywords: MRSA, *mecA*, lateral flow immunoassay, PCR, food safety

1. Introduction

Methicillin-resistant *Staphylococcus aureus* (MRSA) is the most commonly identified antibiotic-resistant pathogen worldwide (Grundmann et al., 2006). MRSA is resistant to several antibiotics, such as methicillin, oxacillin, nafcillin, dicloxacillin, amoxicillin, cephalexin, and meropenem. Thus, this microbe can impose significant challenges to clinical treatment (Green et al., 2012). MRSA was first described in 1961 and spread globally in the early 1990s (DeLeo et al., 2010). MRSA produces heat-stable enterotoxins and is responsible for almost all staphylococcal-associated food poisonings, such as vomiting, retching, diarrhea, and headache. Besides, MRSA can cause several non-food related diseases, including skin and wound infections, pneumonia, and bloodstream infections leading to sepsis or even death (DeLeo et al., 2010). In the United States, MRSA causes about 80,000 life-threatening infections and 11,000 deaths per year, leading to an estimated annual cost of \$10 billion USD. MRSA can be transmitted from infected people through skin-to-skin contact and by digesting contaminated foods (Graveland et al., 2011). Recently, livestock-associated MRSA was isolated from farms and commercial meat products in both the United States and Europe that provoked a worldwide health concern for meat handlers and consumers (Kadariya et al., 2014). Among the research studies during the past decade, the highest prevalence of MRSA from raw meat products was 11.9% in Netherlands (De Boer et al., 2009). Several other studies in the United States identified 2.0-6.6% of retail pork products that carry MRSA (Kadariya et al., 2014). Since food products contaminated with MRSA may not exhibit any spoilage appearance or bad smell, it is challenging for consumers to dispose the contaminated foods prior to consumption. Therefore, it is urgently required to develop an easy-to-use method for rapid and convenient detection of MRSA in agri-food products (e.g., meat).

Methicillin resistance of *S. aureus* is due to the acquisition of the *mecA* gene from staphylococcal chromosome cassette (Archer et al., 1994). The *mecA* gene encodes penicillin binding protein 2A, which

62 has low affinity to β -lactam antibiotics and enables transpeptidase activities in the presence of β -lactams,
63 thus endowing the cells with antibiotic resistance by preventing β -lactams from inhibiting cell wall
64 synthesis (Moellering, 2011; Wu et al., 1996). Therefore, the *mecA* gene has been widely used as a
65 major marker for the characterization of methicillin resistance of *S. aureus* strains (Corrente et al., 2007;
66 Couto et al., 2000; Felten et al., 2002; Salisbury et al., 1997; van Duijkeren et al., 2014).

67 Detection of antimicrobial-resistant foodborne pathogens is critical to quality control of final food
68 products. Conventional methods for the characterization of methicillin resistance of *S. aureus* are time
69 consuming. Several days are required for culture enrichment and isolation, followed by performing
70 minimum inhibitory concentration (MIC) assay that typically takes at least another 24 h. Alternatively,
71 polymerase chain reaction (PCR)-based methods have been developed for sensitive and reliable
72 detection of MRSA (Corrente et al., 2007; Couto et al., 2000; Felten et al., 2002; Salisbury et al., 1997;
73 van Duijkeren et al., 2014). However, the detection and analysis of PCR products are still labor-
74 intensive and require expensive reagents and instrument that are not appropriate for on-site detection.

75 Lateral flow immunoassay (LFI), also called as immunochromatographic strip test, has been
76 recently developed and applied for the detection of foodborne pathogens (Shan et al., 2015). Gold
77 colloidal nanoparticles are widely used in LFI because they can be conjugated with most types of
78 antibodies in a very stable manner and produce visible colors when antibody is combined with its
79 corresponding antigen (Shan et al., 2015). The aim of the current study was to develop a rapid,
80 convenient, and cost-effective method for the detection of *mecA* in MRSA from pork. We performed
81 PCR to amplify *mecA* from the genomic DNA of *S. aureus* with a pair of chemical labeled primers,
82 followed by assembling this nano-colloidal gold-based LFI for the detection of MRSA pure culture. The
83 customized LFI was then applied for rapid detection of MRSA in pork.

84

2. Materials and methods

2.1 Bacterial cultures and genomic DNA preparation

Bacteria were cultivated in 10 mL Giolitti-Cantoni Broth (Beijing Land Bridge Tech Co. Ltd., China) at 37°C overnight with shaking. The overnight bacterial culture (1 mL) was collected and the genomic DNA was prepared using a commercial gDNA extraction kit (Promega, USA) according to the manufacturer's instruction.

2.2 Primer design for polymerase chain reaction (PCR)

The primers for PCR were designed within the highly conserved region of *mecA* using online primer design software (<http://primerexplorer.jp/elamp4.0.0/index.html>) according to the sequence of *mecA* in *S. aureus* (Genbank accession number KC243783.1). Forward primer (5'-TGATGCTAAAGTTCAAAAGAGT-3') and reverse primer (5'-GTAATCTGGAACCTTGTTGAGC-3') (Lu et al., 2013) were labeled with fluorescein isothiocyanate (FITC) and **biotin** at the 5' end, respectively. Both FITC and **biotin** are two typical labels used in numerous molecular biological applications, including immunoassays. The expected amplicon size is **210** bp.

2.3 Polymerase chain reaction (PCR)

The PCR was performed with 1.0 µL genomic DNA templates, 1.0 µL 25 pmol/µL forward primer, 1.0 µL 25 pmol/µL reverse primer, 2.0 µL dNTP (0.25 mM of each nucleotide), 5.0 µL 10× PCR buffer (100 mM Tris-HCl, 500 mM KCl), 3.0 µL 25 mM MgCl₂ and 1.0 µL TaqTM DNA polymerase (5.0 U/µL, TaKaRaTagTM) in a total of 50 µL reaction system. The cycling amplification was conducted on a Bio-Rad T100 Thermocycler (Bio-Rad Laboratories Ltd., Mississauga, Canada) according to the following steps: initiation at 94°C for 3 min, 5 cycles of denaturation at 94°C for 40 sec, annealing at

108 46°C for 40 sec and extension at 72°C for 30 sec, 30 cycles of denaturation at 94°C for 40 sec, annealing
109 at 48°C for 40 sec and extension at 72°C for 30 sec, and final extension at 72°C for 8 min. A 210-bp
110 band can be detected only if *mecA* is present in the genomic DNA. PCR products (5 µL) were separated
111 on a 1.5% agarose gel (Biowest, Nuaille, France) at 100 V for 30 min using Biometra Power Pack P25
112 (Biometra GmbH, Göttingen, Germany), followed by gel staining with Gelred (Biotium, Fremont, USA).
113 Gel imaging was performed using a Bio-Rad Gel Imager XR⁺ System (Bio-Rad Laboratories Ltd.,
114 Mississauga, Canada).

115

116 2.4 *Performance of LFI strip test*

117 LFI strips used in this study were purchased from Ustar Biotech Co. Ltd (Hangzhou, China, cat#
118 0003-03, Patent No. 200610003429.1). The principle of LFI strip test is demonstrated in **Figure 1**. In
119 brief, colloidal gold nanoparticles were conjugated with anti-FITC polyclonal antibody, and then
120 sprayed onto a plain substrate pad for the construction of the sample pad. The test line and control line
121 was coated with anti-biotin antibody and goat anti-rabbit IgG, respectively. An aliquot (3 µL) of PCR
122 product was added onto the sample pad, followed by immersing the sample end of the strip into 97-µL
123 developing buffer (Ustar, Hangzhou, China) in a 1.5-mL Eppendorf tube. The test results became visible
124 in ~3 min.

125

126 2.5 *Detection of S. aureus in pork products using LFI strip test*

127 MRSA strain ATCC 29213 was cultured in Giolitti-Cantoni broth at 37°C overnight to achieve a
128 concentration of about 10⁹ CFU/mL. Then, the culture was washed, ten-fold serially diluted, and
129 inoculated to 25 g pork samples confirmed to be without any bacterial contamination, as determined by

130 the plating assay. Two different concentrations of the spiked samples were prepared, aiming at 2 and 20
131 CFU per 100 g pork product.

132 Twenty five grams of each inoculated pork sample was aseptically transferred to 225 mL sterilized
133 saline water and mixed homogenously using BagMixer® 400VW (Interscience, Breteche, France). The
134 resultant homogenous solution (5 mL) was added into 50 mL 7.5% NaCl broth (Land Bridge Tech Co.
135 Ltd., Beijing, China), followed by incubation with shaking at 37°C for 24 h. A loop of the culture was
136 then streaked onto the surface of Baird-Parker plate (Land Bridge Tech Co. Ltd., Beijing, China). After
137 incubation at 37°C for another 24 h, 10 random colonies with grey to jet-black color surrounded by
138 opaque zone were selected for PCR. Briefly, the colonies were mixed with 200 µL TE buffer (10 mM
139 Tris: 0.1mM EDTA; pH 8.0) and 2 µL lysostaphin (2000 U/mL, Sigma) at 37°C for 30 min, followed by
140 boiling for another 30 min. The solution was centrifuged at 13,000 ×g for 1 min and the supernatant was
141 collected for PCR as aforementioned. An aliquot (3 µL) of the PCR products was detected using LFI
142 strips. In comparison, 5 µL of the PCR products was loaded onto 1.5% agarose gel for electrophoresis
143 and image collection. Each test was performed at least in duplicate. The colonies were further confirmed
144 by Gram staining and coagulase activity test using rabbit plasma (Land Bridge Tech Co. Ltd., Beijing,
145 China).

146 The developed LFI strip test was then applied for routine test of MRSA from 660 frozen pork
147 products imported from Denmark. The test was performed after pre-enrichment as aforementioned and
148 repeated in duplicate.

149

150 2.6 Minimum inhibitory concentration (MIC) test

151 According to the Clinical Laboratory Standards Institute (CLSI) guidelines, ceftiofur MIC test was
152 performed using Mueller-Hinton BBL II agar with different concentrations of ceftiofur (0.5, 1, 2, 4, 8,

153 16, 32, 64 µg/mL). Plates were incubated in ambient air at 37°C. Cefoxitin MICs were read after
154 incubation for 24 h. Quality control strain was methicillin-susceptible *S. aureus* strain ATCC 43300 and
155 the obtained MIC range for cefoxitin was 1~4 µg/mL. According to the CLSI guidelines, MIC value of *S.*
156 *aureus* less than or equal to 4 µg/mL is considered as methicillin susceptible, whereas MIC value higher
157 than 8 µg/mL is regarded as methicillin resistant.

158 3. Results and discussion

159 The detection method described here includes the enrichment of *S. aureus*, preparation of genomic
160 DNA, amplification of *mecA*, and detection by PCR-LFI strip test. PCR amplicons are most commonly
161 analyzed by agarose gel electrophoresis combined with a gel or blot imaging system. In the current
162 study, we detected the PCR amplicons using a colloidal gold LFI strip and compared its specificity and
163 sensitivity to the conventional gel electrophoresis. This colloidal gold LFI strip has comparable
164 specificity but up to 100 times more sensitive than the conventional electrophoresis. We also applied this
165 LFI strip to detect imported pork products contaminated with MRSA and the results were compared to
166 the conventional MIC assay.

167

168 3.1 Specificity of LFI strip test

169 The specificity of the nano-colloidal gold-based LFI strip test was determined using MRSA strain
170 ATCC 29213 and methicillin-susceptible *S. aureus* strain ATCC 43300. Genomic DNA was extracted
171 from these two strains, respectively. PCR amplification was performed as aforementioned and the
172 amplicons were analyzed by both gel electrophoresis and LFI strip. The image of agarose gel is shown
173 in **Figure 2A**, where the expected ~200 bp band is shown in MRSA strain (**Figure 2A**, lane 1).
174 Therefore, our designed primers are specific to methicillin-resistant strain only. In comparison, both the
175 test line and control line on the LFI strip were visible for MRSA strain (**Figure 2B**, lane 1), whereas

176 only the control line was developed for methicillin-susceptible *S. aureus* strain (**Figure 2B**, lane 2).

177 Taken together, the detection results of LFI strip and gel electrophoresis were consistent to each other
178 and had equal specificity.

179

180 3.2 Sensitivity of LFI strip test

181 The sensitivity of this LFI strip test was first analyzed using genomic DNA derived from MRSA
182 strain ATCC 29213. The 10-fold serial dilutions of genomic DNA were used as the templates to amplify
183 *mecA*. An aliquot of 3 μ L and 5 μ L of PCR products was subjected to the analysis by LFI strip and gel
184 electrophoresis, respectively. As shown in **Figure 3A**, the lowest amount of genomic DNA that can be
185 detected by agarose gel was 2 pg/reaction. In comparison, the red test line was clearly visible on the strip
186 by using 200 fg of genomic DNA from MRSA strain ATCC 29213. The test line on the strip was still
187 visible but faint by using 20 fg of genomic DNA. In addition, the amount of DNA amplicon used for
188 LFI strip test was about half of that for gel electrophoresis. Overall, LFI was over 100 times more
189 sensitive than the traditional gel electrophoresis for the detection of DNA amplicons from MRSA.

190 The sensitivity of LFI strip was also evaluated by using artificially contaminated food products.
191 Commercial pork products were first tested for viable bacterial population by conventional plate
192 counting assay and confirmed to be free from *S. aureus*. Then, defined amount of MRSA (*i.e.*, 2
193 CFU/100 g and 20 CFU/100 g) was individually spiked to each pork sample. After bacterial enrichment,
194 LFI strip and gel electrophoresis was then individually applied for the detection of MRSA and the
195 results are shown in **Figure 3B**. In sum, our PCR-LFI assay can detect the presence of MRSA as low as
196 2×10^0 CFU per 100 g of pork products after enrichment at 37°C for 48 h. In the previous studies, the
197 lowest detection limit of MRSA was 48-60 CFU per 100 g of minced beef using gel electrophoresis (De

198 Boer et al., 2009), which was much higher than the detection limit using the LFI strip in the current
199 study.

200 Besides its high sensitivity, LFI strip test has a few other advantages over the conventional agarose
201 gel electrophoresis. Firstly, LFI strip test only takes about 3 min whereas gel electrophoresis requires
202 over 45 min without counting the time for sample loading and imaging. Secondly, LFI strip test does not
203 require the use of expensive reagents or instrument, such as GelRed for gel staining, DNA marker,
204 electrophoresis power pack, and gel imaging system. Thirdly, LFI strip has a control line to evaluate the
205 test performance and provides a baseline color for result interpretation. In contrast, gel electrophoresis
206 does not have a self-control system, which may lead to the lack of clear interpretation of the result.
207 Taken together, PCR-LFI can be considered as a rapid, economic, convenient, and accurate technique
208 for the detection of antibiotic resistance of foodborne pathogenic bacteria.

209

210 3.3 Application of PCR-LFI to detect MRSA from commercial pork products

211 We first isolated 42 *S. aureus* strains from 660 frozen pork products imported from Denmark. By
212 using the PCR-LFI strip test, we identified 7 MRSA strains out of these 42 *S. aureus* strains (**Figure 4**).
213 As recommended by CLSI, one of the standard methods for testing MRSA is the MIC test with agar
214 plate supplemented with cefoxitin. Cefoxitin is recommended for MIC test of MRSA instead of
215 methicillin because methicillin is no longer commercially available in the United States. In addition,
216 cefoxitin is a better inducer of the *mecA* gene (Louie et al., 2000). We conducted MIC test to all of the
217 42 *S. aureus* strains and the results were fully consistent to the PCR-LFI strip test. As shown in **Table 1**,
218 all of the strains with MIC equal to 16 µg/mL or 32 µg/mL were also positive in the PCR-LFI strip test,
219 while the strains with MIC less than 8 µg/mL were negative in the PCR-LFI strip test.

MIC test is widely used to determine antimicrobial resistance, but this method is time-consuming and requires tedious operation (Reller et al., 2009). The sensitivity is related to bacterial growth condition and physiological condition, and thus it can lead to false negative result. PCR is a fast and accurate technology, but gel electrophoresis requires the use of expensive instrument and a large amount of reagents. In contrast, LFI strip test proposed in the current study overcomes these aforementioned disadvantages, demonstrating the feasibility of clinical application in characterizing antimicrobial resistance.

Previous studies reported that all of the *Staphylococcus* strains carrying *mecA* are phenotypically resistant to methicillin, oxacillin, and/or cefoxitin (Corrente et al., 2007; Couto et al., 2000; Felten et al., 2002; Salisbury et al., 1997; Seni et al., 2013; van Duijkeren et al., 2014; Wu et al., 1996). However, not all of the methicillin-resistant *Staphylococcus* strains harbor *mecA* (Martín et al., 2006; Resch et al., 2008). Surprisingly, one recent study identified that there was no correlation between phenotypic methicillin resistance and the presence of *mecA* (Fijałkowski et al., 2016). The authors detected that 50% of *S. vitulinus* isolates from hams and 17% *S. succinus* isolates from chicken cold cuts possessed *mecA*, but none of them showed phenotypic resistance to methicillin (Fijałkowski et al., 2016). Our current study did not identify any discrepancy between methicillin-resistant phenotype and the presence of *mecA* (**Table 1**). Thus, the *mecA*-based PCR-LFI strip assay for the identification of methicillin resistant strains described in the current study is only applicable to *S. aureus* isolates. If this method is applied to an unknown bacterial sample, it can only detect the presence of *mecA* instead of a direct detection for methicillin resistant strains, unless further biochemical assays are performed.

Similar to the current study, another recent work reported the development of a *mecA*-based PCR-LFI assay and applied it for the analysis in clinical samples (Seidel et al., 2016). Our approach has three major advantages than this work. Firstly, the strip that Seidel and coauthors developed was sprayed by

243 neutravidin-labeled carbon nanoparticles, resulting in grey line. In comparison, our strip was sprayed by
244 colloidal gold nanoparticles that displayed pink line and subsequently a better visual judgment. Secondly,
245 the detection limit of our PCR-LFI strip was 2×10^0 CFU/100 g pork product, which was lower than the
246 value (*i.e.*, $2.6 \times 10^1 - 10^2$ CFU per swab assay) reported by Seidel and coauthors. Thirdly, the previous
247 study only performed *mecA*-based PCR-LFI strip to identify the presence of the *mecA* gene, whereas we
248 applied the strip assay to detect MRSA by isolating *S. aureus* first; thus our study is more practical to
249 real sample analysis.

250 In conclusion, *mecA*-based PCR-LFI was successfully applied to distinguish MRSA from
251 susceptible *S. aureus* isolates in commercial pork products. The detection limit of MRSA was 20 fg for
252 genomic DNA, which was significantly lower than the conventional gel electrophoresis. Besides, results
253 can be obtained within 3 min by LFI assay after the amplification of *mecA*. Nevertheless, isolation of *S.*
254 *aureus* strains is required prior to testing their methicillin resistant profile using the PCR-LFI strip.
255 Therefore, further study could be conducted to establish a simultaneous detection method for both *S.*
256 *aureus* strain and the *mecA* gene. In addition, more nucleic acid markers could be developed for quick
257 screening of antimicrobial-resistant pathogens, such as the *aphA1* gene for kanamycin-resistant
258 *Escherichia coli* (Guerra et al., 2003).

259

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331 **Table 1. Summary of the MICs and PCR-LFI test results for 42 isolates of *S. aureus* (n=3).**
332

<i>S. aureus</i> isolates #	MIC (µg/mL)	PCR-LFI*
1	16	+
2	16	+
3	<8	-
4	<8	-
5	16	+
6	<8	-
7	<8	-
8	16	+
9	<8	-
10	<8	-
11	<8	-
12	32	+
13	<8	-
14	<8	-
15	<8	-
16	<8	-
17	<8	-
18	<8	-
19	<8	-
20	<8	-
21	<8	-
22	<8	-
23	<8	-
24	<8	-
25	<8	-
26	<8	-
27	<8	-
28	<8	-
29	<8	-
30	16	+
31	<8	-
32	<8	-
33	<8	-
34	<8	-
35	<8	-
36	16	+
37	<8	-
38	<8	-
39	<8	-

40	< 8	-
41	< 8	-
42	16	+

333

334 * +: positive results; -: negative results.

335

FIGURES

Figure 1. Schematic illustration of lateral flow immunoassay strip used in this study. PCR product labeled with fluorescein isothiocyanate (FITC) and **biotin** was applied to the sample pad (left, grey), bound to gold-α-FITC conjugate, moved laterally towards the absorption pad (right, blue grid) with developing buffer, bound to α-**biotin**, while free gold-α-FITC conjugate bound to α-rabbit IgG, giving positive result with both test line and control line displayed; only free gold-α-FITC conjugate bound to α-rabbit IgG, providing negative result; or no gold-α-FITC conjugate presented at the control line was considered as invalid result.

Figure 2. Comparison of specificity between agarose gel electrophoresis (A) and LFI strip assay (B) for the detection of *mecA* gene. Lane 1, MRSA strain ATCC 29213; Lane 2, methicillin-susceptible *S. aureus* ATCC 43300; Lane 3, sterilized distilled water; Lane M, 100-bp ladder DNA size marker.

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Figure 3. Comparison of sensitivity between agarose gel electrophoresis (top) and LFI strip assay (bottom) for the detection of the *mecA* gene. (A) Ten-fold serial dilutions of genomic DNA (gDNA) was prepared in distilled water and used as templates for PCR. Lane 1, 2 fg; Lane 2, 20 fg; Lane 3, 200 fg; Lane 4, 2 pg; Lane 5, 20 pg; Lane 6, 200 pg; Lane 7, 2 ng; Lane 8, 20 ng; Lane 9, 200 ng; Lane M, 100-bp ladder DNA size marker. (B) Detection sensitivity for MRSA from artificially contaminated pork samples with enrichment (37°C, 48 h). Lane 1, 2 CFU MRSA per 100 g pork product; Lane 2, 20 CFU MRSA per 100 g pork product; PC, MRSA strain ATCC 29213 (positive control); NC, methicillin-susceptible *S. aureus* ATCC 43300 (negative control).

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Figure 4. Application of PCR-LFI assay for the detection of MRSA from imported pork products. Lane 1-7, MRSA positive isolates. Lane 8, MRSA negative *S. aureus* isolate. Lane M, 100-bp ladder DNA size marker; PC, MRSA strain ATCC 29213 (positive control); NC, methicillin-susceptible *S. aureus* ATCC 43300 (negative control).