Fishery Technology 57 (2020) : 141 - 146

Research Note

A Simplified Scheme for Efficient Isolation and Identification of MRSA from Seafood and Aquatic Environment

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Methicillin resistant *Staphylococcus aureus* (MRSA) is a versatile pathogen causing numerous clinical illnesses and a potentially important organism of food borne disease outbreaks. This pathogen has increasingly been detected in several foods including the seafood. However, protocol for efficient isolation and identification in fisheries sector is not developed, which is an environment at elevated salt concentration. Therefore, experiments with 5 enrichment broths and four selective agar plates in different permutations and combinations were tested for improved recovery of MRSA from seafood of retail fish markets, fish landing centers and aquaculture farms. This isolation and identification scheme has been assessed for over 250 samples from the fisheries sector and the results suggested that this is the very simplified procedure for efficient isolation and identification of MRSA and can be accomplished in shortest period of time and as a routine laboratory protocol. This may be adopted in other food sectors after validation. The protocol describes that samples enriched in tryptic soy broth supplemented with 10% salt and 1% sodium pyruvate followed by selective plating in BD ChromAgar MRSA II showed improved isolation of MRSA compared to Oxacillin Resistance Screening Agar Base ORSAB, Baird Parker Agar (BPA) + OX. The protocol also describes the further simplified steps of phenotypic and molecular confirmation of MRSA in shortest period of time.

Received 26 March 2020; Revised 02 April 2020; Accepted 15 April 2020

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#Abbreviations: SP- Sodium pyruvate, TSB- Trypticase soy broth, BPA- Baird parker agar, EgYT – Egg Yolk Potassium tellurite, MSA – Mannitol salt agar, MHB-Mueller Hinton broth, OX – Oxacillin, PRMSB - Phenol red mannitol salt broth, (AZ) Aztreonam, Brain Heart Infusion (BHI), Methicillin- Sensitive *Staphylococcus aureus* (MSSA), Methicillin-Resistant *Staphylococcus aureus* (MRSA).

MRSA is considered as dreadful in nature since they harbour pathogenic determinants as well as antibiotic resistance (Liu, 2009). The MRSA is multidrug resistant in nature (resistance to three or more than three class or group of antibiotics) (Magiorakos et al., 2012). Since 2010 it is being isolated from seafood (Atyah et al., 2010; Hammad et al., 2012; Sergelidis et al., 2014; Visnuvinayagam et al., 2015; Murugadas et al., 2016b). Although *S. aureus*/ MRSA is not a common microflora of fish, its presence indicates contamination from water or ice and due to anthropogenic activity indicating the poor sanitary condition. Even though protocol is developed for the screening of MRSA from clinical samples (Brown et al., 2005; CDC, 2017), for surveillance programme (Weese, 2007; Bortolami et al., 2017), there is no approved protocol from international bodies for the isolation of MRSA from foods that include seafood. Incidence of MRSA in food and food producing animals varied significantly because of the variation in the methods, sample collection scheme, sample types, enrichment protocol used etc., (Weese, 2010). Considering the difficulties and criticality involved in the isolation of MRSA from fishery environment, we present herewith a simplified procedure for efficient recovery of MRSA, confirmation up to molecular level from seafood and aquatic environment at a shortest period of time in comparison to several protocols available in public domain.

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Bruins and others observed hindrance in recovering epidemic clones of MRSA from clinical samples in media with above 2.5% salt concentration (Bruins et al., 2007), contrary MRSA clones associated in fishery sector generally have high salt tolerance. Considering the complex microbial diversity associated with the fish and fishery products and its environs, we made comparison of several enrichment, selective primary and secondary plating media for enhanced recovery/detection of Methicillin resistant/sensitive *Staphylococcus aureus* from seafood.

Control strains: *S. aureus* ATCC 43300 oxacillin resistant and *S. aureus* ATCC 29213 oxacillin sensitive strains were employed.

Initially the procedure of standardization of enrichment and selective plating was carried out in permutations and combinations of media published elsewhere for clinical sector, food producing animals. **Broth1:** TSB + 10% NaCl + 1% SP, **Broth 2:** PRMSB + 3% NaCl + Az 10 µg mL^{-1} OX 2 µg mL^{-1}, **Broth 3:** TSB 3% NaCl 1% SP AZ 10 µg mL^{-1} OX 2 µg mL^{-1}, **Broth 4:** TSB 6% NaCl AZ 10 µg mL^{-1} OX 2 µg mL^{-1}, **Broth 5:** PRMSB 6% AZ 10 µg mL^{-1} OX 2 µg mL^{-1}. Selective plating media: BPA EgYT OX 2 µg mL^{-1}, MSA OX 2 µg mL^{-1}, ORSAB, BD ChromAgar MRSA II, Biorad MRSA Select.

The simplified stepwise procedure for improved isolation and identification of MRSA from seafood and aquatic environment is depicted in the Fig. 1 which is a harmonized scheme based on several studies. The scheme proposed can identify pure MRSA from seafood and fishery environment samples by the end of five days. If the study envisaged only presence or absence in a given sample, then the protocol may be reduced to three days. This simplified scheme is limited to seafood and fishery environment samples. However, this may be adapted to other food producing animals.

Day 1: Step1: Enrichment step: Take 10 g portion of minced seafood sample aseptically and add to 90 mL Tryptic soy broth supplemented with 10% salt and 1% sodium pyruvate in stomacher bags (BA6141, Seward, UK) and homogenize it in a stomacher blender for 30 s at 230 rpm (Seward 400 circulator, UK). Transfer to sterile 250 mL conical flask (method modified from AOAC, 2012; Bennett & Lancette, 2001) and incubate overnight at 35ºC (Critical, should not be more than 35ºC).

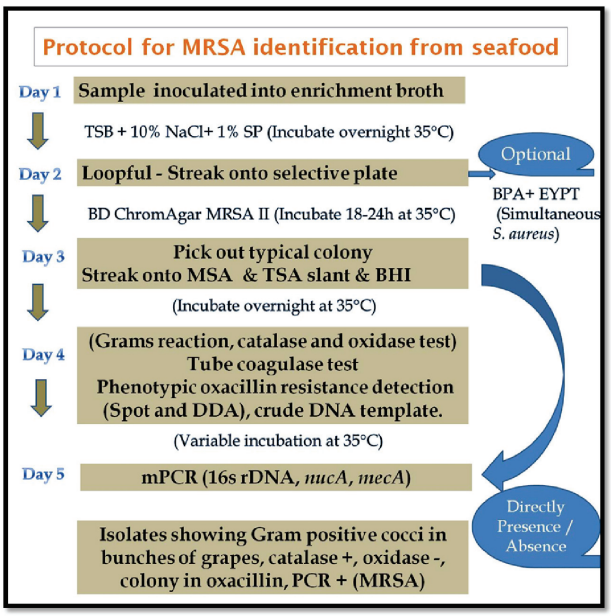

Fig. 1. TSB- Trytpic soy broth, SP- Sodium pyruvate, BPA - Baird parker agar, EYPT - Egg Yolk Potassium Tellurite, MSA- Mannitol salt agar, TSA - Tryptic soy agar, BHI - Brain heart infusion, DDA - Disk diffusion assay, mPCR- multiplex PCR

Day 2: Step2: Selective plating: Streak a loopful of culture from enrichment broth onto BD ChromAgar MRSA II (Cat. No.215228) and optionally to confirm the presence of *S. aureus* in the sample, streak on to Baird Parker agar supplemented with Egg yolk potassium tellurite. Incubate BD ChromAgar MRSA II at 35ºC for 24 h and BP agar plates at 35ºC for 36 to 48 h.

Look for the characteristic *S. aureus*/ MRSA colonies *viz.*, mauve coloured in BD ChromAgar MRSA II & circular, smooth, convex, moist gray to jet-black colour, 2-3 mm in diameter with light-colored (off-white) margin surrounded by opaque zone and outer clear zone from Baird Parker agar and pick out characteristic colony from respective agar for further confirmation. If the study requires only for presence or absence confirmation of MRSA/*S. aureus* directly proceed for molecular confirmation step.

Day 3: Step3: Identification: Streak the colonies picked out from the respective selective enrichment medium to Mannitol Salt agar media and look for medium sized white/yellow colony with yellowish discoloration of surrounding media. Proceed further for other characteristics such as morphological (Grams reaction) and biochemical tests (catalase test, oxidase test, glucose and mannitol fermentation,

and coagulase test) as described by FDA (Bennett & Lancette, 2001; 2016).

Check the culture for purity in TSA agar plate for individual colony and store the culture in TSA slant with liquid paraffin overlay for short term preservation. Stab inoculation in TSA semisolid media with liquid paraffin overlay or cryopreservation as glycerol stock in -80ºC ultra freezer for long term preservation.

Note: Other cultures on BPA such as *Staphylococcus epidermidis* will produce - No growth to fair growth; small, colorless to gray-brownish colonies; no clear zones. *Proteus mirabilis* produces - No growth to good growth; dark brown colonies; swarming reduced.

Bacteria other than MRSA on BD ChromAgar MRSA II may utilize other chromogenic substrates in the medium resulting in blue to blue/green colored colonies or if no chromogenic substrates are utilized, the colonies appear as white or colorless.

Day 4: Step 4: Simultaneously perform all these tests with the young presumptive MRSA/ *S. aureus* culture from slant or MSA plates.

Biochemical identification: Initially perform Grams staining, catalase test and oxidase test with the presumptively identified culture. Gram positive cocci in bunches of grapes, catalase positive and oxidase negative cultures should be taken for coagulase test.

Tube coagulase test: Transfer suspected cultures stored in TSA into small tubes containing 0.2-0.3 ml BHI broth and incubate BHI culture suspension for 18-24 h at 35ºC. Reconstitute the dried EDTA coagulase rabbit plasma with sterile distilled water. Add 0.5 ml of reconstituted plasma to the BHI culture and mix thoroughly. Incubate the tube in water bath at 35ºC and examine periodically for every hour for up to 4 h and 24 h period for clot formation. Only firm and complete clot that stays in place when tube is tilted or inverted is considered positive for *S. aureus*/ MRSA. Partial clot should be tested further (Sperber and Tatini, 1975; Bennett & Lancette, 2001; 2016).

Phenotypic oxacillin resistance confirmation: Oxacillin resistance in *S. aureus* is confirmed by agar dilution method as per CLSI (2017) or simply called as Oxacillin agar screening method. Perform the test in MHA with 4% NaCl and oxacillin added at 6 µg/mL concentration of media. Take a colony or young culture from the slant and suspend in normal saline diluents and adjust the turbidity to 0.5 McFarland standard. Make grid lines or divide into quadrants and spot inoculate 1-µL in each grid and incubate at 35ºC. Read the plate in transmitted light by 24 h. Look for at least a minute colony or light film of growth at spot inoculated area for detecting oxacillin resistance. *S. aureus* ATCC 29213 (MSSA) and *S. aureus* ATCC 43300 (MRSA) were used as oxacillin negative and positive quality control strains respectively.

Additionally perform Disk diffusion assay in MHA as per Bauer et al. (1966) with cefoxitin (30 µg disk) and incubate at 35°C for 16–18 h. The results to be interpreted as per CLSI (2017). Zone diameter of ≤ 21 mm were considered as *mecA* positive while zone diameter of ≥ 22 mm were considered as *mecA* negative. *S. aureus* ATCC 29213 (MSSA) and *S. aureus* ATCC 43300 (MRSA) were used as oxacillin negative and positive quality control strains respectively.

Crude template DNA preparation by Boiling lysis method: Take 1mL of overnight grown culture from TSB and transfer to sterile 1·5-mL microfuge tubes in order to obtain a turbid suspension of bacteria (~ 1–2 × 10^9 cells mL^{-1}). Centrifuge the tube at 10000 rpm for 15 mins. Discard the supernatant. Add 500 mL of 1 X TE buffer pH 8 and vortex it. Once again centrifuge at same speed. If required proceed for one more washing with 1 X TE buffer. Finally keep the microfuge tube containing the cell suspensions in 500 mL of 1 X TE buffer pH 8 in a boiling water-bath or dry heat bath for 10 min to lyse the cells, then immediately chill on ice or deep freezer. Leave the tube at -20ºC until use. Before use quick spin at maximum speed of 15000 rpm to pellet the debris and transfer the supernatant to fresh sterile tube. Store the supernatant containing crude DNA at -20ºC until use (Oliviera et al., 2014).

Multiplex PCR (mPCR) assay targeting 16S rRNA (*Staphylococcus* genus specific), *nuc* (*S. aureus* species specific), and *mecA* (a determinant of methicillin resistance) genes developed by Zhang et al. (2004) is adapted in this simplified scheme. Here in this scheme target for mupirocin resistance determination is dropped.

Prepare the PCR master mix for every 25 µL reactions *viz.,* 22 µl PCR mixture containing 50 mM

KCl, 20mM Tris-HCl (pH 8.4), 2.5 mM $MgCl_2$, 0.2mM each deoxynucleoside triphosphate (dATP, dUTP, dGTP, and dCTP) (Thermofisher scientific, USA), 0.12 µM each 16S rRNA and *mecA* primers, 0.04 µM each *nuc* primer and 1.0 U of *Taq* DNA polymerase Recombinant (Thermofisher scientific, USA). Distribute 22 µl to each reaction tubes and add crude DNA template (3 µl) finally. The entire procedure of setting up of PCR reaction need to be carried in chilled condition or in coolant or ice box. Keep the PCR tube reaction mixture for Amplification in Thermal cycler.

The details of primers are *sta750F* - AAC TCT GTT ATT AGG GAA GAACA, *sta750R* CCA CCT TCCTCC GGT TTG TCA CC, *nucF* GCG ATT GAT GGT GAT ACG GTT, *nucR* AGC CAAGCC TTG ACG AAC TAA AGC, *mecA*F GTA GAA ATG ACT GAA CGTCCG ATA A, *mecA*R CCA ATT CCA CAT TGT TTC GGT CTAA as described by Zhang et al. (2004). Cycling conditions should be 94°C for 5 min, followed by 10 cycles of 94°C for 40 s, 58°C for 40 s, and 72°C for 1 min; Followed by 25 cycles of 94°C for 1 min, 50°C for 1 min, and 72°C for 2 min; and 72°C for 10 min. Keep the reagent controls (without template DNA), a positive control (*S. aureus* ATCC 43300) and a negative control (*S. epidermidis* ATCC 1113).

Prepare 3% agarose gel containing 0.5 µg of ethidium bromide mL^{-1} of required volume to suit to the need for the comb and boat for agarose gel electrophoresis. Take 10 µl aliquots of PCR product and mix with 2 µl 6X Gel loading dye and load onto well in the agarose gel. Perform electrophoresis (90 V for 90 min for bigger gel), visualize the gel after electrophoresis in Gel Documentation system or Transilluminator. The expected amplicon size are 750 bp for 16s rDNA, 280 bp for *nuc* gene, 320 bp for *mecA* gene. The multiplex targets are very close to each other and hence, use 50bp DNA ladder for the better interpretation.

In this study, we observed that trypticase soy broth with 10% salt and 1% sodium pyruvate as the best enrichment in combination with BD ChromAgar MRSA II. Subsequent screening in Mannitol salt agar as secondary screening plates to rule out other non-mannitol salt utilizing *Staphylococcus*. Optionally if the study envisaged to identify simultaneously *S. aureus* also then Baird Parker agar with egg yolk potassium tellurite may be included in the selective plating.

Isolates which showed typical mauve coloured colony form BD ChromAgar MRSA II & circular, smooth, convex or moist gray to jet-black colour, 2-3 mm in diameter with light-colored (off-white) margin surrounded by opaque zone and outer clear zone in Baird Parker agar are further screened for white or yellow colony with yellow discolouration of the media in mannitol salt agar. In this step the colony may be taken for purity check and stored in TSA slants. The cultures which showed Gram positive reaction (cocci appearing in bunches of grapes) and catalase reaction positive and oxidase reaction negative are generally *Staphylococcus* spp. Drop the culture from analysis if oxidase test positive (*Staphylococcus sciuri*, *Staphylococcus lentus*, *Staphylococcus vitulinus* and *Staphylococcus fleurettii*) (Bannerman, 2003). Isolates which showed characteristic in other three tests above and coagulase test positive and growth in Oxacillin agar screen test are considered as Methicillin Resistant *S. aureus*. However, to rule out the other coagulase positive *Staphylococcus* (*Staphylococcus aureus*, *S. intermedius*, *S. schleiferi* subsp. *coagulans*, *S. hyicus*, *S. lutrae*, *S. delphini*, and *S. pseudintermedius*) from *S. aureus* perform multiplex PCR for differentiation of *mecA* mediated Methicillin Resistant Staphylococci, MRSA and other MSSA strains (Zhang et al., 2004). Isolates which showed amplification in the 16s rDNA region specific for *Staphylococcus* sp (756 bp), *mecA* specific for methicillin resistance (324 bp), and *nuc* gene for nuclease gene (280 bp) are confirmed as MRSA.

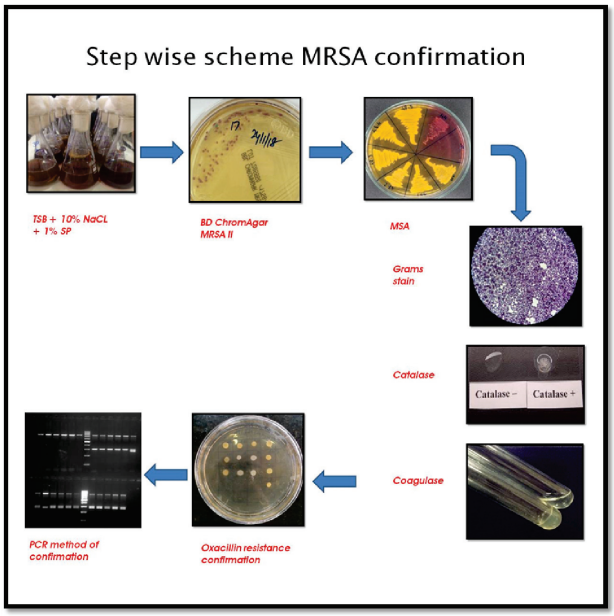

Fig. 2. Stepwise Results of MRSA identification scheme

Majority of the cases Oxacillin agar screen tests are *mecA* gene positive MRSA. However, include the possibility of observation of *mecC* strains or Oxacillin resistant *mecA* negative MRSA if exclusively works in Livestock associated environment. The results of entire scheme–are depicted in Fig. 2.

Troubleshooting:

In some occasions, the isolates may not show positive in *mecA* gene in multiplex PCR.

- Look for expiry date of the BD ChromAgar, whether isolation step is performed with media of more than 15 days from the of date of expiry. The colonies observed in the media may be β-lactamase hyper producers. In this condition perform disk diffusion assay with Penicillin G and Nitrocefin test for detection of β-lactamase producers (CLSI, 2017).
- Perform PCR for *mecC* for identification of methicillin resistance mediated by *mecA* variant called *mecC* (García-Alvarez *et al.,* 2011). This condition may be attributed to livestock associated strains.
- Recently *mecA* negative Oxacillin resistant *S. aureus* were detected in animal samples, here cefoxitin disk diffusion assay gives consistent result.
- Deep red colonies observed in BD ChromAgar MRSA II or colonies observed after 24 h should not be picked.
- In case of BPA, medium sized colonies with halo zone appeared first and white precipitate after 24 h around the colony generally leads to *S. aureus.* Colonies with more precipitate and very big sized halo before 24 h are not *S. aureus.*

Hence, this scheme will be efficient enough to detect MRSA from the fisheries sector in hyper saline environments. The detection can be performed in a time frame of minimum 3 days to a maximum of 5 days which is very useful for the industry involved in the seafood production. For further molecular characterization to the clone level and determination of antimicrobial resistance *viz.,* staphylococcal protein A (spa) typing, multi locus sequence typing (MLST) and other procedures, the researchers may use the published data (Murugadas et al., 2016a; 2016b; Murugadas et al., 2017a; 2017b and Murugadas et al., 2019).

Acknowledgements

The authors are thanked to Indian Council of Agricultural Research, New Delhi for the support and the Director, ICAR-Central Institute of Fisheries Technology, Cochin for encouragement.

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