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EVALUATION OF EFFICACY OF TESTS RECOMMENDED BY A PrPN EN ISO 11290–1:1999 STANDARD FOR IDENTIFICATION OF *LISTERIA* SPP. AND *LISTERIA MONOCYTOGENES* ISOLATED FROM MEAT AND MEAT-PROCESSING ENVIRONMENT

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The efficacy of confirmatory tests recommended by a PrPN EN ISO 11290-1:1999 standard for identification of *Listeria* spp. and *L. monocytogenes* strains in food products was examined in our studies. Confirmatory assays consisted of catalase and motility (a characteristic 'umbrella--shape' outgrowth) tests for *Listeria* spp. as well as hemolytic activity and sugar fermentation (D-xylose and L-rhamnose) tests for *L. monocytogenes*. They were compared with results of *multiplex* PCR (polymerase chain reaction) designed for confirmation of genus and species. Seventy strains were tested. In the great majority (52 strains, 74.3%) they were of beef and pork carcasses origin. Eighteen strains (25.7%) were collected by swabbing in meat-processing environment. All presumptive strains were motile in ambient temperature, catalase-positive, presented hemolytic activity and characteristic morphological features, which enabled to classify them as *L. monocytogenes*. The comparison of pathogen identification results carried out with the standard tests and genetic analysis revealed that 84.3% of results was conformable (45 strains recognized as *L. monocytogenes* and 14 strains identified as *Listeria* spp. using both procedures). Variations referred to over 15% of results. Nine strains (12.9%) were identified as *L. monocytogenes* based on the sugar fermentation pattern whereas not confirmed by *multiplex* PCR. The affiliation of two strains (2.8%) identified as *L. monocytogenes* by *multiplex* PCR was not confirmed in the sugar fermentation test. The number of inaccurately classified strains in tests recommended by ISO standard highlights its limited efficacy for identification of *L. monocytogenes* strains.

INTRODUCTION

Listeria monocytogenes is one of the most serious 'emerging' pathogens in raw meat production, handling and manufacturing, distribution and consumption of meat products. It poses a potential health hazard to consumers due to registered epidemic and sporadic cases of listeriosis linked to the ingestion of such foods [Jay, 1996; Rocourt *et al.*, 2000]. Studies gave clear evidence that food of meat origin is frequently and highly contaminated with this pathogen [Kwiatek, 1993; Wendlandt & Bergann, 1994; Manzano *et al.*, 1997; Dąbrowski *et al.*, 1999; Paziak--Domańska *et al.*, 1999]. The level of its contamination is determined especially by sanitary condition of abattoirs and processing plants, mainly due to effortless colonization of such environments by selected *L. monocytogenes* strains. Adaptation and survival of selected strains in exceptionally unfavourable and artificial conditions are promoted by particular strain features. Ascribed to biofilm formation, *L. monocytogenes* is a microorganism highly resistant to applied disinfecting procedures. It is one of the main reasons that its entire elimination from abattoir and processing plant environments is practically impossible [Archer, 1990; Blackmann & Frank, 1996]. To reduce the level of the contamination observed in production environments and to protect quality of meat and meat products, it is necessary to provide and implement monitoring systems for particular stages of production, *i.e*. HACCP (Hazard Analysis and Critical Control Points).

Conventional methods of *Listeria* isolation and identification based on cultures in/on enrichment and/or selective media followed by analyses of morphological features (*e.g*. a colony appearance) and biochemical activity (*e.g*. a hemolytic activity, esculin hydrolysis) are time-consuming and take at least 4–5 days to be completed [Manzano *et al.*, 1997]. Results of species identification carried out by traditional methods are often not reliable due to visual similarity of *Listeria* spp. colonies on selective media (false--positive results) or problems with isolation of bacteria affected *e.g*. by temperature stress (false-negative results) [Norton & Batt, 1999; Norton *et al.*, 2001]. Among many novel techniques adapted for detection and identification of *L. monocytogenes*, techniques based on polymerase chain reaction (PCR) enable highly specific, sensitive and quick analyses [Manzano *et al.*, 1997].

Results of identification of *Listeria* spp. and *L. monocytogenes* strains carried out with the Polish standard confirmatory tests and *multiplex* PCR were compared in this studies. The comparison of results obtained using both methods based on analysis of phenotypic and genetic traits, respectively, was aimed to evaluate reliability and efficacy of officially recommended tests in routine microbiological identification of *L. monocytogenes* present in the meat--processing environment.

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MATERIALS AND METHODS

Bacterial strains and culture conditions. Seventy *Listeria* spp. strains were subjected to analysis (Table 1). The majority (52 strains, 74.3%) was collected by swabbing selected points of halved beef and pork carcasses. Eighteen strains (25.7%) were isolated from the processing environment, equipment and machines (a carving knife, skinning machine, cold-room door handle and floor). All strains were isolated from a meat-processing plant in the Western Pomeranian region of Poland during the winter season, 2001.

Isolates from the environment and meat carcasses were obtained by swabbing a 25-cm^2 area with sterile disposable swabs and cultures were conducted according to the PrPN EN ISO 11290-1:1999 standard. They were incubated in 10 mL of half-Fraser broth (Oxoid, England) at 30°C for 24 h. Then, 0.1 mL of the broth was transferred onto *Listeria* Selective Agar (LSA, Oxoid) and incubated at 37°C for 48 h. Colonies grown on LSA were preliminarily identified based on their phenotypic features (a characteristic colony appearance and ability to esculin hydrolysis). Subsequent confirmatory tests for *Listeria* genus included a catalase activity test (a suspension of material from selected, single colony in a drop of hydrogen peroxide) and a motility test.

Motility of strains was tested in motility agar tubes (0.35%) at 25°C incubated up to 7 days. The test was slightly modified by supplementing the medium with 1% of 2,3,5-triphenyltetrazolium chloride (TTC) to facilitate reading and interpretation of results. The characteristic red 'umbrella shape' outgrowth was considered to be a positive result (Figure 1).

To confirm whether a strain belonged to *L. monocytogenes* species or not, the hemolytic activity was tested on blood agar (nutrient agar supplemented with 4% of human blood) incubated at 37°C and checked after 24 h and 48 h. *L. monocytogenes* strains created a narrow transparent zone (β-hemolysis) around a colony.

FIGURE 1. A characteristic and distinctive red 'umbrella-shape' outgrowth of *Listeria* in a motility agar tube supplemented with 1% of 2,3,5-triphenyltetrazolium chloride (TTC) incubated at ambient temperature for 7 days.

Next, sugar fermentation tests were conducted. Strains were cultured in soy broth enriched with yeast extract (TSYEB, Oxoid) and incubated at 25°C for 24 h. When the turbidity appeared, a loop of the culture was reinoculated into broth supplemented with particular carbohydrate solutions (L-rhamnose or D-xylose). A significant change of medium colour from violet to yellow during a 5-day incubation at 35–37°C was considered as a positive result (a medium acidification). According to the standard a result: D-xylose (-) and L-rhamnose (+), is typical of *L. monocytogenes* strains.

DNA extraction and *multiplex* PCR. DNA extraction and genus and species identification using *multiplex* PCR was conducted as described previously [Mędrala *et al.*, 2003].

RESULTS

All isolates tested were catalase-positive, motile in ambient temperature and hemolytically active. Results of identification based on the sugar fermentation and hemolytic activity were consistent with the PCR results for the majority of strains (84.3%). A few strains (12.9%) classified as *L. monocytogenes* based on the sugar fermentation: D-xylose (-), L-rhamnose (+) were not confirmed in *multiplex* PCR. Two strains (2.8%) were negative in fermentation tests but were identified as *L. monocytogenes* by the PCR technique. Results obtained are presented in Tables 1 and 2.

DISCUSSION

Monitoring of food-processing areas is of crucial importance to control and avoid *L. monocytogenes* transmission *via* food to consumers. PrPN EN ISO 11290-1:1999 recommends four identification tests based on *Listeria* spp./ /*L. monocytogenes* expression of phenotypic traits. Results of two final tests (hemolytic activity, sugar fermentation) are considered to be sufficient to determine ultimate affiliation of the analyzed strain to *L. monocytogenes* species.

All *Listeria* spp. should be catalase-positive and demonstrate ability to form 'umbrella shape' in motility

TABLE 1. Characteristics of *Listeria* spp. strains used in the studies.

No. of strains tested	Source of isolation	Catalase activity	Motility at 25°C	β-hemolysis	Fermentation		*multiplex* PCR
					D-xylose	L-rhamnose	
6	carving knife	+	+	+	-	+	*L. monocytogenes*
8	skinning machine	+	+	+	-	+	*L. monocytogenes*
3	cold-room door handle	+	+	+	-	+	*L. monocytogenes*
1	floor	+	+	+	-	+	*L. monocytogenes*
10	pork carcass	+	+	+	-	+	*L. monocytogenes*
9	pork carcass	+	+	+	-	+	*Listeria* sp.
10	pork carcass	+	+	+	+	-	*Listeria* sp.
2	pork carcass	+	+	+	+	-	*L. monocytogenes*
4	beef carcass	+	+	+	+	+	*Listeria* sp.
17	beef carcass	+	+	+	-	+	*L. monocytogenes*

TABLE 2. Comparative results of *L. monocytogenes* identification carried out using tests recommended by PrPN EN ISO 11290-1:1999 and *multiplex* PCR.

No. of strains	Catalase test	β-hemolysis	Sugar fermentation	*multiplex* PCR	(%)
45	+	+	+	+	64.3
14	+	+	-	-	20.0
9	+	+	+	-	12.9
2	+	+	-	+	2.8

(+) results characteristic of *L. monocytogenes*; (-) results suggesting different than *L. monocytogenes* affiliation of a tested strain

agar if incubated in ambient temperature. Yet, the presence of catalase-negative strains was recorded earlier by Bubert *et al.* [1997]. In routine practice the interpretation of results of motility tests in motility agar tubes incubated at ambient temperature in particular raised serious doubts, generally caused by problems with a medium standardization. The medium is frequently too solidified or too liquid depending on the agar brand (not specified in the standard) at the same concentration recommended (0.35%). Supplementation of medium with tetrazolium salt (TTC), which in its oxidized form is colourless and soluble and if reduced becomes red and insoluble, proved to be a beneficial solution. *Listeria* spp. strains use TTC as an electron acceptor and reduce it to formazan. The outgrowth of strains in the modified medium is observed earlier due to the quick colour change along the stab line. The red 'umbrella' is more distinctive and easier to interpret but the addition of TTC does not accelerate its manifestation. However, we strongly suggest that the non-fatiguing enrichment of medium with 2,3,5--triphenyltetrazolium chloride may facilitate evaluation of strain motility in case if such tests are necessary.

To complete strain species status, hemolysis activity and sugar fermentation tests are applied. Problems with interpreting results of the hemolytic activity in our studies were eliminated by a blood agar standardization and replacement of sheep blood by fresh human blood. Thanks to it, the necessity of using the CAMP test with *Staphylococcus aureus* and *Rhodococcus equi* to potentiate weak or doubtful β-hemolytic reaction, proven to demonstrate ambiguous results by Johnson and Lattuada [1993], was excluded.

Our results also revealed that final *L. monocytogenes* confirmatory tests recommended by the PrPN EN ISO 11290-1:1999 standard based on sugar fermentation of two sugars were not sufficiently quick, sensitive and specific. Tests on L-rhamnose and D-xylose fermentation frequently gave puzzling spontaneous coloured reactions whose interpretation might differ depending on the staff member conducting analyses and reading the results. It is not surprising as even results of standardized biochemical tests including 7 sugars (API®*LISTERIA*, bioMérieux) were regarded as not too specific and unfailing [Mędrala *et al.*, 2002]. Sugar fermentation tests usually require repetitions for dubious strains and extend time of analysis what definitely should be avoided not only during routine control of food quality and/or contamination of processing lines but principally in the case of epidemiological threat.

Results of the above-mentioned tests based on analysis of phenotypic traits were compared with *multiplex* PCR test focused on detection of individual gene fragments conservative for genus and species. It revealed that over 15% of strains were erroneously identified based on their ability to ferment selected carbohydrates. Strains indicated eventually by fermentation tests as potentially pathogenic--to-a-consumer *L. monocytogenes* (12.9%) were not confirmed with *multiplex* PCR. In practice it may end in unnecessary financial losses for food producers who are forced to recall such products baselessly. In addition, a few strains (2.8%), whose affiliation to *L. monocytogenes* species was eliminated in sugar fermentation tests, turned out to belong to the species based on *multiplex* assays. It may lead to a situation when products contaminated with *L. monocytogenes*, whose ingestion may pose a health hazard to consumers, may be permitted to distribution and consumption if decision is based only on the results of tests recommended by the standard. Therefore, it is strongly advisable to implement alternative-for-classical, more sensitive and specific tests as referential methods for routine diagnostics of actual food pathogen contamination.

Multiplex PCR technique applied in our studies may play such a role. It is one of the simplest varieties of PCR but recently it has turned out to be evidently functional for fast and sensitive detection of *L. monocytogenes* in the food industry, including meat-processing environments [Bansal *et al.*, 1996; Bubert *et al.*, 1999; Wesley *et al.*, 2002]. In our case, adaptation of protocol proposed by Bansal *et al.* [1996] enabled to ascertain simultaneously if a particular strain belonged to *Listeria* genus and whether it was identified as *L. monocytogenes* or not. Therefore, one PCR reaction performed may give an inside view on contamination of the monitored environment with *Listeria* spp. and provides information about distribution of *L. monocytogenes* known to be potentially pathogenic to humans. Assuming that confirmatory tests for *L. monocytogenes* recommended by the PrPN EN ISO 11290-1:1999 allow up to 5 days of waiting for the results as in the case of sugar fermentation assays, application of *multiplex* PCR may result in significant time reduction with the test completed within 10–12 h.

CONCLUSIONS

1. Specificity and sensitivity of the recommended *multiplex* PCR assay together with a short time of analysis and its unambiguous results suggest that it should be included as a reference method to confirm species and genus identification of presumptive *Listeria* spp. isolates.

2. Compared with *multiplex* PCR results, 15.7% of strains were erroneously identified using tests recommended by PrPN EN ISO 11290-1:1999 what emphasizes their uselessness in routine diagnostics of *L. monocytogenes* strains.

3. Supplementation of motility agar tubes with 1% of 2,3,5-triphenyltetrazolium chloride (TTC) in the motility test facilitates interpretation of results recommended by PrPN EN ISO 11290-1:1999 (the characteristic red 'umbrella--shape' outgrowth).

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OCENA SKUTECZNOŚCI TESTÓW ZALECANYCH PRZEZ NORMĘ PrPN EN ISO 11290-1:1999 DO IDENTYFIKACJI SZCZEPÓW *LISTERIA* SPP. I *LISTERIA MONOCYTOGENES* IZOLOWANYCH Z SUROWCA I ŚRODOWISKA PRZETWÓRSTWA MIĘSNEGO

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W pracy badano skuteczność testów potwierdzających, zalecanych w normie PrPN EN ISO 11290-1:1999 dla identyfikacji szczepów *Listeria* spp. i *L. monocytogenes* w produktach żywnościowych. Testy potwierdzające obejmowały: dla *Listeria* spp. – test na wytwarzanie katalazy, test na zdolność ruchu (wzrost w kształcie charakterystycznego parasola); dla *L. monocytogenes* – test na hemolizę, testy na zdolność rozkładu cukrów (D-ksyloza i L-ramnoza). Wyniki identyfikacji porównywano z rezultatami analizy *multiplex* PCR zaprojektowanej do jednoczesnego potwierdzania przynależności rodzajowej i gatunkowej szczepu. Przeanalizowano 70 szczepów, z czego większość – 52 (74,3%) uzyskano z wymazów z półtusz wieprzowych i wołowych, a 18 (25,7%) wyizolowano z wymazów ze środowiska przetwórstwa mięsnego (tab. 1). Wszystkie domniemane szczepy poddane identyfikacji były ruchliwe w temperaturze pokojowej, wykazywały właściwości hemolityczne, aktywność katalazy oraz prezentowały typowe cechy morfologiczne pozwalające na zakwalifikowanie ich do gatunku *L. monocytogenes*. Porównanie rezultatów identyfikacji patogenu testami normy ISO i analizą genetyczną wykazało, że 84,3% wyników było zgodnych (45 szczepów rozpoznano jako *L. monocytogenes*, podczas, gdy 14 zostało z tego gatunku wykluczonych przy użyciu obu procedur) (tab. 2). Rozbieżności dotyczyły ponad 15% wyników. Dziewięć szczepów (12,9%) zostało zidentyfikowanych jako *L. monocytogenes* przez test oparty na rozkładzie cukrów, a wykluczonych przez test genetyczny. Dwa szczepy (2,8%) oznaczone jako *L. monocytogenes* przez *multiplex* PCR nie były nią według wzoru fermentacji cukrów. Liczba szczepów błędnie rozpoznanych na podstawie testów zgodnych z normą ISO wskazuje na ich ograniczoną przydatność do identyfikacji szczepów *L. monocytogenes*.