A selective chromogenic plate, YECA, for the detection of pathogenic Yersinia enterocolitica: specificity, sensibility and capacity to detect pathogenic Y. enterocolitica from pig tonsils

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Abstract

A new selective chromogenic plate, YECA, was tested for its specificity, sensitivity and accuracy to detect pathogenic Y. enterocolitica from pig tonsils. We tested a panel of 26 bacterial strains on YECA and compared it to PCA, CIN and YeCM media. Detection of pathogenic Y. enterocolitica was carried out on 50 pig tonsils collected in one slaughterhouse. Enrichment was done in PSB and ITC broths. Streaking on YECA and CIN was done in direct, after 24H incubation of ITC, after 48H incubation of PSB and ITC. All the plates were incubated at 30°C during 24 hours. Presence of typical colonies on CIN and YECA was checked and isolates were biotyped. Pathogenic Y. enterocolitica strains showed an important growth on YECA with small and red fuchsia colonies while biotype 1A exhibited very few violet colonies. Enrichment in ITC during 48H gave the best performance for detecting positive samples in pathogenic Y. enterocolitica and YECA could detect directly pathogenic Y. enterocolitica strains (2, 3 and 4). Combination of ITC enrichment and YECA detection generates a time-saver by giving a positive test for pathogenic Y. enterocolitica in 72 hours.

Introduction

In 2009, yersiniosis was, for the sixth consecutive year, the third most frequently reported human zoonosis in the Europe, with a total of 8,354 confirmed cases (EFSA, 2010). In France and most other countries worldwide, biotype 4 is the most prevalent biotype isolated from humans (69%), followed by biotype 2 (30%) and biotype 3 (Savin and Carniel, 2008). Pigs are considered the principal reservoir for the types of Y. enterocolitica pathogenic to humans. Pigs do not develop clinical signs, but they do carry Y. enterocolitica in their oral cavity, on tongues and tonsils, and in lymph nodes, and excrete this bacterium in their feces (Thibodeau et al., 1999). Detection of Yersinia is carried out by using ISO 10273-2003 method. This method is recommended for both food and pig tonsil analyses (EFSA, 2007) but involves time-consuming enrichment steps in two broths, PSB and ITC, followed by plating on 2 selective media, SSDC and CIN (De Boer, 1992). Biochemical tests on isolates are also necessary to confirm Yersinia and to determine the biotypes.
In this work, we tested a new selective chromogenic plate, Yersinia enterocolitica agar (YECA), for its specificity, sensitivity, and accuracy to detect pathogenic Y. enterocolitica from pig tonsils.

Material and Methods

Specificity of YECA. The specificity of YECA (AES Chemunex, Combourg, France) was tested against 26 strains listed in table 1. Each culture was streaked on CIN agar plate, on YeCM medium (Weagant, 2008) and, on YECA plates. If growth of bacteria was observed on plate, importance of growth in a scale from 1 to 5 and characteristics of the colonies were noted.
Sensitivity of YECA. Y. enterocolitica strains from biotype 1A, 2, 3, and 4 were incubated in 5ml of BHI broth during 24h at 30°C. A 10-fold serial dilution of the cultures was done and 100µl of each dilution were spread on YECA and compared with PCA, CIN and YeCM media. Enumeration of the colonies was then performed after incubation of the plates at 30°C for 24hours.
Detection of pathogenic Yersinia enterocolitica from pig tonsils. 50 pig tonsils were collected from a slaughterhouse. From each tonsil, 10 g were cut in small pieces and put into a bag containing 90 ml of PSB broth. After stomaching, 10µl were streaked directly onto YECA and CIN plates; and 1 ml was transferred in 9 ml of ITC broth. PSB and ITC were incubated at 25°C for 48 hours, before a second streaking onto YECA and CIN. In addition, after 24 hours of enrichment in ITC broth, an extra streaking on YECA and CIN was performed. All the plates were incubated at 30°C for 24 hours. Presence of typical colonies on CIN and on YECA was checked. At least two typical colonies per plate were streaked on

YeCM and these plates were incubated at 30°C for 24 hours. This step on YeCM permitted to differentiate rapidly the pathogenic Y. enterocolitica (red bull's-eye-like colonies) from the non-pathogenic Y. enterocolitica (blue-purple colonies). Confirmation and biotyping was then done by biochemical assays as described in ISO 10273:2003 standard.

Results

The 3 pathogenic Y.enterocolitica showed an important growth with small and red fuchsia colonies on YECA. Growth of biotype 1A was much reduced with violet colonies and absence of growth or growth with non typical colonies was observed for the other strains (table 1). The other Yersinia-likes strains were able to growth on YECA but the number of colonies was very small. For the 14 non-Yersinia strains, we observed for CIN, YeCM and YECA an absence of growth or growth but as not characteristic colonies on these media. Numeration of pure culture of Y.enterocolitica strains on YECA was similar to those realised on PCA, CIN and YeCM, except for biotype 1A. For this biotype, colonies on YECA could be numerated only at the dilutions -1, -2, -3 while on PCA, CIN and YeCM, it was possible to count the colonies until the dilution -8.

Out of the 50 tonsils, pathogenic Y. enterocolitica were detected on CIN and YECA respectively from 17 and 15 tonsils after direct streaking, from 21 and 22 tonsils after ITC-24H, from 28 and 28 tonsils ITC-48H, and from 8 and 5 tonsils after PSB-48H. Enrichment in ITC for 48H gives the best performance for detecting positive samples and same number of positive samples was obtained from CIN and YECA. However, YECA compared to CIN could detects directly pathogenic Y. enterocolitica strains (2, 3 and 4) while CIN does not differentiate the biotype 1A from the pathogenic biotypes. A total of 141strains were collected on YECA and biotyped. Among the 141 strains, 135 were identified as biotype 4, two as biotype 3 and four as biotype 2. YECA is able to detect these 3 pathogenic biotypes from naturally contaminated pig tonsils. In addition, the ITC- YECA way generates a time-saver by giving a positive test in 72H.

Discussion

The ISO 10273-2003 standard is the reference method for isolating Y. enterocolitica from foods. This method is also recommended for pig tonsils analysis (EFSA, 2007) but involves time-consuming enrichment steps in two broths, PSB and ITC, followed by plating on 2 selective media, SSDC and CIN (De Boer, 1992). Van Damme et al. (2010) showed that the use of a two-day incubation period at 25°C, instead of five days, for the PSB broth resulted in a significantly higher recovery rate of Yersinia. Wauters et al. (1988) indicated that enrichment in PSB broth gave better results for non-pathogenic strains, whereas enrichment in ITC broth gave better results for pathogenic strains. The SSDC agar (Wauters et al. 1988) does not always allow differentiating Yersinia from interfering flora such as Morganella, Proteus, Serratia and Aeromonas. The medium CIN (Schiemann et al., 1979) is highly selective but Citrobacter freundii, Enterobacter agglomerans and the species of Aeromonas and Klebsiella produce colonies of similar morphology (Harmon et al., 1983). However, users recognized that detection on CIN agar is easier since Y. enterocolitica has relatively more characteristic colony morphology on this medium compared to SSDC (Fondrevez et al. 2010). This author recommends the use of CIN after the enrichment in ITC broth; tested on 900 pig tonsil swabs, this way recovered a larger number of positive samples (14.0% versus only 9.1% with the modified ISO method). These media, CIN and SSDC, moreover lack the ability to differentiate potentially virulent Y. enterocolitica from the non-pathogenic strains and other Yersinia. Only panel of biochemical tests (esculin hydrolysis, indole production, and fermentation of xylose and trehalose) as described in the ISO 10273:2003 method permits to identify the biotype. Recently, Weagant (2008) has developed a chromogenic medium (YeCM) for the specific detection of Y. enterocolitica. On this medium, pathogenic Y. enterocolitica strains grow as red bull's-eye-like colonies while non-pathogenic Y. enterocolitica grows as blue-purple colonies. Fondrevez et al. (2010) proposed its use after the CIN step to quickly discriminate the non pathogenic biotype from the pathogenic biotypes.

YECA showed a real capacity to favour the growth of the pathogenic Y. enterocolitica (Biotype 2, 3 and 4) with typical colonies, small and red fuchsia. Growth of biotype 1A was much reduced with violet colonies. Moreover, YECA exhibits a stronger inhibitor effect on the growth of the Yersinia-like strains while numerous colonies were observed on the chromogenic media YeCM. Numeration of pure culture of Y. enterocolitica strains on YECA was similar to those carried out on PCA, CIN and YeCM, except for biotype 1A for which high inhibition was observed.

When tested from naturally contaminated pig tonsils, we observed a best performance for detecting positive samples after enrichment in ITC than in PSB, and we obtained similar percentage of positive samples between CIN and YECA after enrichment in ITC during 48 hours. This result is consistent with the work of Fondrevez et al. (2010). Because CIN does not differentiate biotype 1A from the pathogenic biotypes, isolates were confirmed as Yersinia and biotyped by biochemical assays as described in ISO method. All the isolates from YECA were however identified as pathogenic Y.

enterocolitica strains. It could be possible to isolate the three pathogenic biotypes 2, 3, and 4 on YECA after ITC enrichment; biotype 4 representing 95.7% of all isolates.

Conclusion

We have described a simplified method that efficiently detects pathogenic Y. enterocolitica in pig tonsils and that it is less time-consuming than the ISO 10273: 2003 standard. In three days, it was possible to detect pathogenic Y. enterocolitica strains from pig tonsils when using YECA after ITC. Combination of ITC enrichment and YECA detection generates a time-saver by giving a positive test for pathogenic Y. enterocolitica in 72 hours. Moreover use of YECA could decrease the need for biochemical tests for confirmation and biotyping.

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Table 1: Growth and color of colonies of strains used to test the specificity of YECA media.

Strains obtained from	Name of the strains	Growth* and color of colonies on CIN plate	Growth and color of colonies on YeCM plate	Growth and color of colonies on YECA plate
Yersinia RNC from Pasteur Institute (Paris, France)	*Yersinia enterocolitica* biotype 2 (IP383)	+++++ red with a translucent rim	+++++ red bull's-eye-like	+++++ small red fuchsia
	Yersinia enterocolitica biotype 3 (IP29228)	+++++ red with a translucent rim	+++++ red bull's-eye-like	+++++ small red fuchsia
	Yersinia enterocolitica biotype 4 (IP134)	+++++ red with a translucent rim	+++++ red bull's-eye-like	+++++ small red fuchsia
	Yersinia enterocolitica biotype 1A (IP124)	+++++ red with a translucent rim	+++++ blue-purple	+ violet colonies (5)
Collection of the Pasteur Institute (Paris, France)	*Yersinia aldovae* (CIP103162)	+++++ red with translucent rim	+++++ yellow/red with translucent rim	+ small red fuchsia (1)
	Yersinia bercovieri (CIP103323)	+++++ red with translucent rim	+++++ yellow/red with translucent rim	++ yellow/small red fuchsia
	Yersinia frederiksenii (CIP80.29)	+++++ red with translucent rim	+++++ blue to green	++ green/small red fuchsia
	Yersinia kristensenii (CIP80.30)	+++++ red with translucent rim	++++ red with translucent rim	++ pink//small red fuchsia
	Yersinia massilliensis (CIP109351)	+++++ red with translucent rim	+++++ green	++ green/small red fuchsia
	Yersinia mollaretii (CIP103324)	+++++ red with translucent rim	+++++ yellow/red with translucent rim	+ small red fuchsia (1)
	Yersinia rohdei (CIP103163)	+++++ red with translucent rim	+++++ yellow/red with translucent rim	+ pink (1)
	Yersinia ruckeri (CIP82.80)	No growth	No growth	No growth
Collection of the Pasteur Institute (Paris, France)	*Salmonella* Typhimurium (CIP55.43)	No growth	No growth	No growth
	Campylobacter jejuni (CIP70.2)	No growth	No growth	No growth
	Enterococcus feacalis (CIP55/42)	No growth	No growth	No growth
	Lactobacillus plantarum (CIP103151)	No growth	No growth	No growth
	Pseudomonas fluorescens (CIP525)	+++++ yellow	+++++ yellow	+ pink
	Brochothrix thermosphacta (CIP103251)	No growth	No growth	No growth
Field strains from Anses collection	*Listeria monocytogenes*	No growth	No growth	No growth
	Escherichia coli	No growth	No growth	No growth
	Staphylococccus aureus	No growth	No growth	No growth
	Klebsiella sp.	No growth	No growth	No growth
	Proteus mirabilis	No growth	No growth	No growth
Strains from Fondrevez *et al.*, (2010)	*Morganella morganii*	+++++ yellow	+++++ yellow	++ yellow/pink
	Pseudomonas sp.	+++++ yellow	+++++ yellow	+ pink
	Serratia liquefaciens	+++++ pink with translucent rim	+++++ green	++++ green/blue/pink

**Growth was measured from no growth (absence of colonies) to +++++ (important culture with numerous colonies).*
Yersinia enterocolitica strains were purchased from Dr. E. Carniel from the Yersinia RNC (Pasteur Institute, Paris, France)