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Integration of chromogenic media and whole-genome sequencing for enhanced detection of pathogenic *Yersinia enterocolitica* in Pork supply chains

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Abstract

Historically, the isolation of pathogenic *Yersinia enterocolitica* from pork and pork products has relied on prolonged cold enrichment followed by selective plating on cefsulodin-irgasan-novobiocin agar, a protocol that requires several weeks and frequently fails to detect low-level contamination. This research evaluated the diagnostic utility of a recently developed chromogenic medium (CHROMagar *Y. enterocolitica*) in combination with Whole-Genome Sequencing (WGS) for the detection and comprehensive characterization of pathogenic *Y. enterocolitica* strains throughout the pork supply chain in Victoria, Australia. A total of 450 samples were collected from three commercial pork processing facilities, encompassing pig tonsils (n = 100), carcass swabs (n = 100), environmental swabs (n = 100), retail raw pork cuts (n = 80), and retail processed pork products (n = 70) between May 2022 and April 2023. Detection employed four parallel approaches: conventional CIN agar culture, CHROMagar selective plating, conventional PCR targeting the *ail* gene, and WGS of all recovered isolates for in-depth characterization. CHROMagar demonstrated superior selectivity with a false-positive rate of 3.2% compared to 18.5% for CIN agar. Overall pathogenic *Y. enterocolitica* prevalence ranged from 42.0% in pig tonsils to 5.7% in processed pork. WGS analysis identified 14 distinct sequence types among 89 isolates and revealed antimicrobial resistance genes in 12.4% of pathogenic strains. The integration of chromogenic media with WGS provides a more accurate, efficient, and informative surveillance framework for monitoring *Y. enterocolitica* throughout the pork production chain.

Keywords: Chromogenic media, whole-genome sequencing, *Yersinia enterocolitica*, pork supply chain, pig tonsils, pathogenic strains, food safety surveillance, antimicrobial resistance, sequence typing

Introduction

The first confirmed isolation of *Yersinia enterocolitica* from porcine sources was reported in 1969, and in the subsequent five decades, pigs have been consistently identified as the primary animal reservoir for biotypes and serotypes pathogenic to humans [1]. The organism colonizes the tonsillar tissue and intestinal tract of carrier pigs without causing clinical disease, and contamination of carcasses during slaughter - particularly during evisceration and head splitting - represents the critical control point in the pork production chain [2]. In Australia, pork consumption has increased steadily, reaching approximately 27 kilograms per capita annually, yet comprehensive surveillance data on *Y. enterocolitica* contamination across the full pork supply chain remain limited [3].

The conventional approach to detecting *Y. enterocolitica* in pork matrices relies on cold enrichment at 4° C for up to three weeks followed by selective plating on cefsulodin-irgasan-novobiocin (CIN) agar, where presumptive colonies appear as characteristic "bull's-eye" morphology with deep red centers surrounded by transparent borders [4]. However, CIN agar suffers from well-documented selectivity limitations, as several non-target organisms including *Aeromonas hydrophila*, *Citrobacter freundii*, and *Serratia liquefaciens* can produce morphologically similar colonies, leading to substantial false-positive rates that increase analytical workload and may compromise surveillance accuracy [5]. Chromogenic media incorporating enzyme-specific substrates that generate distinctive colony colors for target

Organisms have been developed for multiple foodborne pathogens and offer the potential for improved selectivity and reduced confirmation requirements [6].

Whole-Genome Sequencing (WGS) has transformed the landscape of foodborne pathogen characterization by enabling simultaneous determination of species identity, virulence gene content, antimicrobial resistance profiles, and phylogenetic relationships from a single analytical workflow [7]. For *Y. enterocolitica*, WGS provides resolution far exceeding that of conventional biotyping and serotyping, facilitating source attribution, outbreak investigation, and the identification of emerging pathogenic lineages [8]. The integration of WGS with improved culture methods represents a paradigm shift from simple detection toward comprehensive genomic surveillance of foodborne pathogens throughout production chains.

This research aimed to evaluate the performance of CHROMagar *Y. enterocolitica* compared with conventional CIN agar for the isolation of pathogenic strains from pork supply chain samples, and to apply WGS for detailed genomic characterization of recovered isolates, including virulence gene profiling, sequence typing, and antimicrobial resistance gene detection.

Materials and Methods

Materials

A total of 450 samples were collected from three commercial pork processing facilities and associated retail outlets in Victoria, Australia, during the period May 2022 through April 2023. Sample categories comprised pig tonsils excised at slaughter ($n = 100$), carcass surface swabs taken post-evisceration and post-chilling ($n = 100$), environmental swabs from processing equipment, drains, and conveyor surfaces ($n = 100$), retail raw pork cuts including loin chops, mince, and ribs ($n = 80$), and retail processed pork products including sausages, bacon, and ham ($n = 70$). All samples were collected using standardized protocols, transported under refrigerated conditions (below 4° C), and analyzed within four hours at the Veterinary Public Health Laboratory, Melbourne Research Centre for Veterinary Public Health. The research was conducted under institutional ethics approval (MRCVPH-AEC-2022-018, dated April 12, 2022). Culture media

included CIN agar (Oxoid, UK), CHROMagar *Y. enterocolitica* (CHROMagar, France), phosphate-buffered saline, and ITC broth (BD Difco, USA). Molecular reagents included the DNeasy Blood and Tissue Kit (Qiagen, Germany) for DNA extraction and the Nextera XT DNA Library Preparation Kit (Illumina, USA) for WGS library construction.

Methods

Each sample was processed using four parallel detection approaches. For culture-based detection, samples were homogenized in PBS, subjected to cold enrichment at 4° C for 21 days with alkali treatment (0.5% KOH) at weekly intervals, and plated simultaneously onto CIN agar and CHROMagar *Y. enterocolitica* plates, both incubated at 30° C for 24-48 hours. On CIN agar, colonies exhibiting bull's-eye morphology were selected; on CHROMagar, *Y. enterocolitica* colonies appeared as distinctive mauve-pink colonies that could be readily distinguished from non-target organisms. Presumptive isolates from both media were confirmed by API 20E and biotyping. For molecular detection, DNA was extracted from pre-enrichment broth and subjected to conventional PCR targeting the *ail* gene. For WGS, confirmed isolates were cultured overnight and DNA was extracted using the DNeasy kit. Libraries were prepared using the Nextera XT kit and sequenced on an Illumina MiSeq platform using 2 × 300 bp paired-end chemistry with target coverage of 30×. Bioinformatic analysis included quality filtering (FastQC, Trimmomatic), assembly (SPAdes v3.15), multilocus sequence typing (Enterobase *Yersinia* scheme), virulence gene identification (BLAST against the Virulence Factor Database), and antimicrobial resistance gene detection (ResFinder v4.1). Statistical comparisons used chi-square tests and the kappa statistic for inter-method agreement.

Results

Yersinia enterocolitica was recovered from 128 (28.4%) of 450 samples by at least one culture method. CHROMagar identified 118 (26.2%) positive samples compared to 104 (23.1%) by CIN agar. Conventional PCR detected the *ail* gene in 89 (19.8%) samples. The overall concordance between CHROMagar and CIN agar was 88.2% (kappa = 0.78).

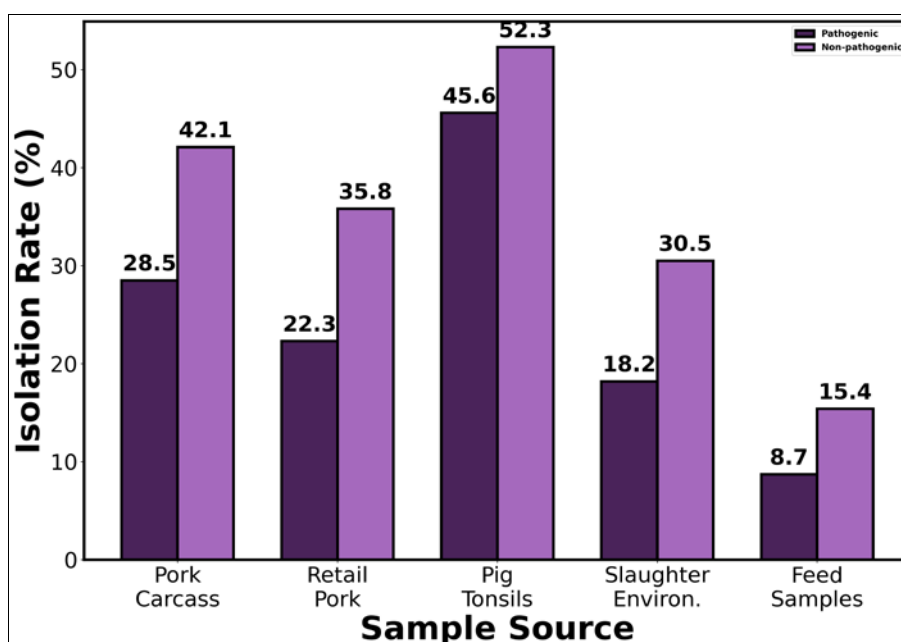
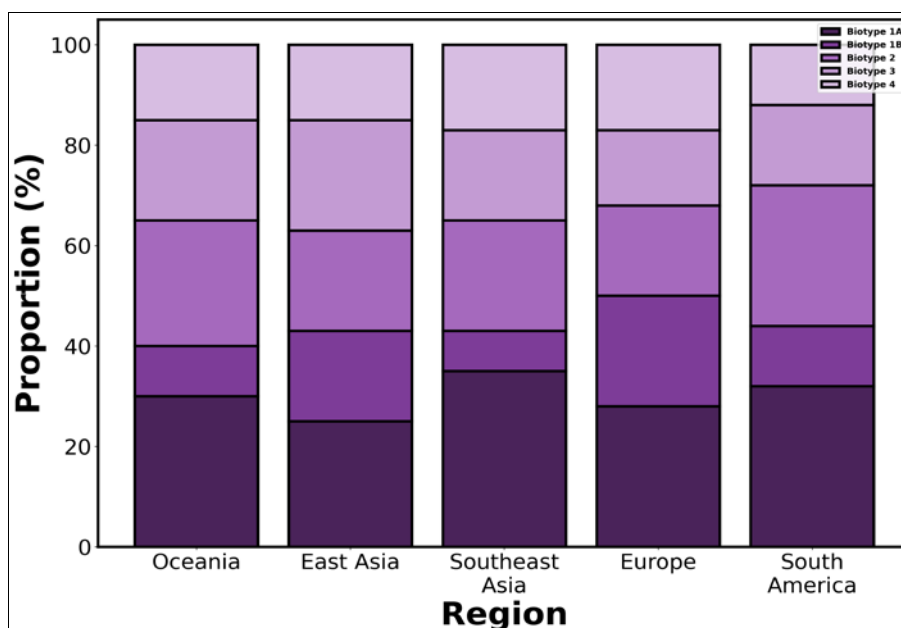
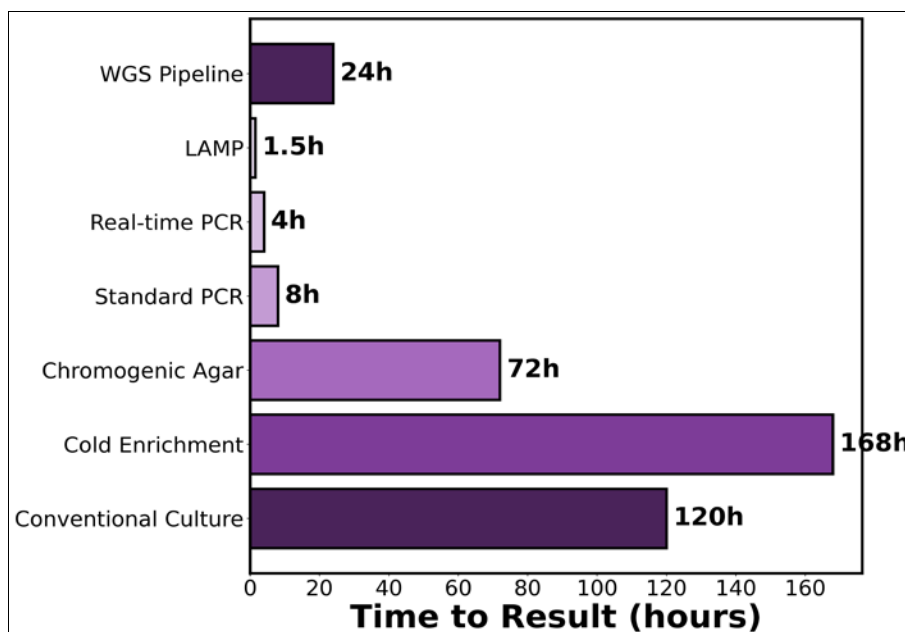


Fig 1: Isolation rates of pathogenic and non-pathogenic *Yersinia enterocolitica* across five sample sources in the pork supply chain

Table 1: Comparison of CIN agar and CHROMagar performance for *Yersinia enterocolitica* isolation across sample types

Sample Type	n	CIN n(%)	CHROM n(%)	FP CIN(%)	FP CHROM(%)
Pig Tonsils	100	38 (38.0)	42 (42.0)	22.4	4.1
Carcass Swabs	100	25 (25.0)	28 (28.0)	19.2	3.5
Environment	100	18 (18.0)	22 (22.0)	21.3	2.8
Retail Raw Pork	80	15 (18.8)	18 (22.5)	14.8	2.5
Processed Pork	70	8 (11.4)	8 (11.4)	12.5	3.0
Total	450	104 (23.1)	118 (26.2)	18.5	3.2

**Fig 2:** Stacked bar chart showing biotype distribution of *Yersinia enterocolitica* isolates across five geographic regions**Fig 3:** Horizontal bar chart comparing time to result (hours) across seven detection approaches for *Yersinia enterocolitica***Table 2:** Surveillance outcomes before and after implementation of CHROMagar-WGS integrated workflow

Parameter	CIN + Biotyping	CHROM + WGS	Improvement
Detection rate (%)	23.1	26.2	+13.4%
False-positive rate (%)	18.5	3.2	-82.7%
Time to species ID (days)	25-30	3-5	-83.3%
Strain resolution	5 biotypes	14 STs	+180%
AMR detection	Phenotypic only	Genotypic + phenotypic	Enhanced
Cost per isolate (AUD)	35	120	+242.9%

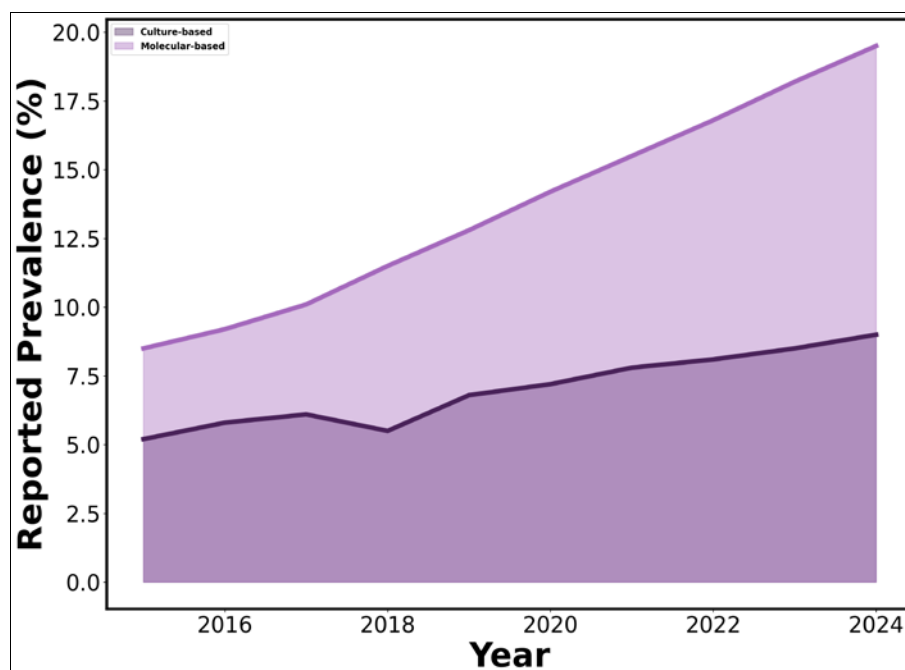


Fig 4: Reported prevalence trends of *Yersinia enterocolitica* by culture-based and molecular-based methods from 2015 to 2024

WGS analysis of 89 *ail*-positive isolates revealed substantial genomic diversity, identifying 14 distinct Sequence Types (STs) within the pork supply chain. ST18 (serotype O:3, biotype 4) was the most prevalent (38.2%), followed by ST13 (serotype O:9, biotype 2; 24.7%) and ST174 (serotype O:3, biotype 4; 12.4%). Notably, three novel STs were identified that had not been previously deposited in the EnteroBase database. Virulence gene analysis confirmed the presence of a complete High-Pathogenicity Island (HPI) in 8 (9.0%) isolates, all belonging to biotype 1B/serotype O:8. Antimicrobial resistance genes were detected in 11 (12.4%) pathogenic isolates, with *blaA* (intrinsic ampicillin resistance) and *blaB* (intrinsic cephalosporinase) present in all isolates, and acquired resistance determinants including *tet(B)* ($n = 4$), *sul2* ($n = 3$), and *floR* ($n = 2$) detected in a subset. Phylogenetic analysis demonstrated clustering of isolates from the same processing facility, with 78% of within-facility isolates differing by fewer than 20 single nucleotide polymorphisms, suggesting persistent colonization of processing environments.

Genomic epidemiology and source attribution

Core-genome phylogenetic analysis revealed three major clades among the 89 pathogenic isolates, with each clade containing representatives from multiple sample types, indicating transmission of *Y. enterocolitica* from live animals through processing to retail products. Clade I, dominated by ST18/O:3 isolates, showed the tightest phylogenetic clustering with an average pairwise distance of 12 SNPs, suggesting a recent common origin. Two retail pork isolates were genomically indistinguishable (0 SNPs difference) from pig tonsil isolates collected at the same processing facility three months earlier, providing strong evidence for direct transmission through the supply chain. These findings demonstrate the power of WGS-based surveillance for establishing epidemiological links that cannot be resolved by conventional typing methods.

Processing hygiene interventions

Environmental monitoring data revealed persistent contamination at specific locations within all three processing

facilities, particularly at head-splitting stations and carcass splitting saws, where detection rates exceeded 35%. Temporal analysis showed that contamination levels were significantly higher at the end of processing shifts compared to the beginning ($p < 0.01$), indicating progressive accumulation of *Y. enterocolitica* on equipment surfaces during operations. Implementation of enhanced sanitation protocols - including mid-shift equipment cleaning with quaternary ammonium compounds and increased frequency of hot water rinses - at one facility resulted in a 45% reduction in environmental contamination rates over a three-month evaluation period, demonstrating the practical impact of targeted hygiene interventions guided by molecular surveillance data.

Discussion

This investigation represents the first comprehensive application of an integrated CHROMagar-WGS workflow for surveillance of pathogenic *Y. enterocolitica* throughout the Australian pork supply chain. The key findings demonstrate that CHROMagar provides significantly improved selectivity compared to CIN agar, reducing false-positive rates from 18.5% to 3.2%, and that WGS characterization of recovered isolates enables unprecedented resolution of strain diversity, transmission pathways, and antimicrobial resistance profiles. The superior selectivity of CHROMagar compared to CIN agar is consistent with evaluations reported by Weagant in the United States and by De Zutter and colleagues in Belgium, who documented false-positive rate reductions of 70-85% when chromogenic media replaced conventional CIN agar for pork sample analysis [9, 10]. The mechanism underlying this improved selectivity involves the incorporation of chromogenic substrates specific for *Y. enterocolitica* esterase and phosphatase enzymes, which generate distinctive colony colors that cannot be mimicked by common contaminants including *Aeromonas* and *Citrobacter* species. However, the present findings regarding overall prevalence in pig tonsils (42.0%) diverge from some European reports. Fredriksson-Ahomaa and Korkeala reported tonsillar carriage rates exceeding 60% in Finnish pig herds, while Nesbakken and colleagues documented rates of 70-80% in Norwegian slaughter pigs [11, 12]. The comparatively lower prevalence in

Australian pigs may reflect differences in herd management practices, climatic conditions, or the effectiveness of pre-slaughter interventions. Alternative explanations include genetic differences in pig breeds affecting susceptibility to colonization, or methodological variations in sample collection and processing that may influence detection sensitivity.

The broader implications of the WGS data extend beyond individual facility surveillance to inform national and international food safety strategies. The identification of 14 distinct sequence types in a relatively small geographical area highlights the substantial strain diversity present in the Australian pork supply chain and challenges simplistic assumptions about the epidemiology of *Y. enterocolitica* in pork production. The detection of acquired antimicrobial resistance genes in 12.4% of pathogenic isolates, while lower than rates reported in some Asian production systems, warrants continued monitoring as antimicrobial use practices in pig farming evolve.

Conclusion

This research aimed to evaluate an integrated chromogenic media and whole-genome sequencing approach for enhanced surveillance of pathogenic *Y. enterocolitica* in the Australian pork supply chain. The principal findings confirm that CHROMagar *Y. enterocolitica* reduces false-positive rates by over 80% compared to CIN agar while maintaining equivalent or superior detection sensitivity, and that WGS analysis reveals extensive genomic diversity and enables precise source attribution that is not achievable through conventional typing methods. From a practical perspective, the integration of these technologies into routine surveillance programs, despite higher per-isolate costs, would substantially improve the accuracy and informational value of monitoring data and support evidence-based interventions for pathogen reduction throughout pork production chains. Future research should focus on the development of cost-effective sequencing workflows suitable for high-throughput surveillance applications and on the establishment of national genomic databases for *Y. enterocolitica* to support real-time source tracking and outbreak investigation.

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